Université de Sherbrooke

The inhibition of Bid expression by Akt leads to resistance to TRAIL-induced apoptosis in ovarian cancer cells

By

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Thesis is presented to the Faculté de Médecine et des Sciences de la Santé in order to obtain the Diploma of Master of Sciences (M.Sc.) in Microbiology

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RÉSUMÉ

Mécanismes de la résistance intrinsèque à l'apoptose induite par TRAIL chez les cellules de cancer ovarien

par

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Mémoire présenté à la Faculté de Médecine et des Sciences de la Santé en vue de l'obtention du diplôme de maître ès sciences (M.Sc.) en Microbiologie, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

Le cancer épithélial ovarien (CEO) est la principale cause de décès parmi les cancers gynécologiques. La majorité des patientes sont diagnostiquées à un stade tardif de la maladie. La survie de ces patientes est limitée à cause de la récurrence de la maladie devenue résistante à la chimiothérapie. Le développement de nouveaux agents thérapeutiques pour le traitement de ce cancer est ainsi une priorité importante dans le domaine afin d'améliorer le taux de survie. La cytokine TRAIL ("TNF-related apoptosis-inducing ligand") est un nouvel agent prometteur pour le traitement du cancer, incluant le cancer ovarien, car il a la capacité unique de déclencher l'apoptose dans les cellules cancéreuses et d'épargner les cellules normales.

Précédemment, notre laboratoire a montré qu'un nombre significatif de lignées cellulaires dérivées de cancer ovarien et de cellules provenant de cultures primaires de cancer ovarien sont intrinsèquement résistantes à l'apoptose induite par le TRAIL. Les mécanismes menant à la résistance intrinsèque sont largement inconnus.

Les cellules résistantes au TRAIL montrent souvent une activation accrue de la voie de survie PI3K/Akt. En se basant sur les observations que les ascites provenant de cancer ovarien induisent une activation de la voie PI3K/Akt chez les cellules de cancer ovarien sensibles au TRAIL, résultant en une inhibition de l'apoptose produite par le TRAIL, nous proposons l'hypothèse que l'activation de la voie PI3K/Akt dans les cellules CEO joue un rôle important dans la résistance intrinsèque à l'apoptose induite par le TRAIL.

Les objectifs de mon projet sont de démontrer qu'Akt est impliqué dans la régulation de l'apoptose induite par le TRAIL dans les cellules CEO et de déterminer les mécanismes par lesquels Akt contribue à la résistance au TRAIL.

Nous avons démontré que l'activation d'Akt réduit la sensibilité des cellules CEO au TRAIL. Les cellules résistantes au TRAIL furent sensibilisées à l'apoptose induite par le TRAIL via traitement avec des inhibiteurs de la voie PI3K ou Akt. L'inhibition de la voie PI3K/Akt n'a pas interférée avec le recrutement et l'activation du pro-caspase-8 au DISC (death-inducing signaling complex). Parallèlement, une surexpression stable d'Akt1 dans les cellules sensibles au TRAIL a résulté en une certaine résistance au TRAIL. Malgré le fait que la pro-caspase-8 était recrutée et activée au DISC dans les lignées cellulaires

résistantes et sensibles, le clivage de Bid par la caspase-8 apparaissait seulement dans les lignées sensibles au TRAIL. L'activation d'Akt dans les cellules sensibles au TRAIL a inhibé le clivage de Bid induit par TRAIL. De plus, l'expression de Bid fût diminuée par l'activation d'Akt. La déplétion de Bid par siRNA dans les cellules de CEO sensibles au TRAIL fût associée à une baisse de l'apoptose médiée par TRAIL et une surexpression de Bid dans les cellules résistantes au TRAIL résultât en une hausse de l'apoptose médiée par TRAIL.

Dans leur ensemble, ces résultats suggèrent qu'Akt est un facteur critique pour la résistance intrinsèque chez les cellules CEO et qu'un mécanisme important par lequel l'activation d'Akt contribue à la résistance au TRAIL, est la régulation de l'expression de la protéine pro-apoptotique Bid.

Suite à ces données, nous spéculons que l'activation d'Akt pourrait être un biomarqueur potentiel pour prédire la réponse des patientes à une thérapie basée sur TRAIL et que l'inhibition de la voie PI3K/Akt pourrait devenir une des stratégies pour surmonter la résistance à TRAIL dans le cancer ovarien.

Mots clés : cancer ovarien épithélial; TRAIL; apoptose; résistance; Bid.

SUMMARY

Mechanisms of intrinsic resistance to TRAIL-induced apoptosis among ovarian cancer cells

By

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Mémoire is presented to the Faculté de Médecine et des Sciences de la Santé in order to obtain the Diploma of Master of Sciences (M.Sc.) in Microbiology, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke,
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EOC (epithelial ovarian cancer) is a leading cause of death from gynecological cancers. The majority of the patients with EOC are diagnosed at a late stage. The survival of these patients is limited due to recurrence of chemotherapy resistant disease. Therefore, the development of novel therapeutic agents for the treatment of EOC is urgently needed and it is a high priority in the field. TNF-related apoptosis-inducing ligand (TRAIL) is a promising novel agent for the treatment of cancer, including EOC, because of its unique ability to trigger apoptosis in cancer cells and spare normal cells.

Our laboratory has previously shown that a significant number of EOC cell lines and primary EOC samples are intrinsically resistant to TRAIL-induced apoptosis. The mechanisms leading to intrinsic resistance are largely unknown. TRAIL-resistant cells often display increased activation of the pro-survival PI3K/Akt pathway. Based on our observations that EOC ascites induced activation of the PI3K/Akt pathway in TRAIL-sensitive EOC cells which resulted in inhibition of the TRAIL-mediated apoptosis, we hypothesized that activation of the pro-survival PI3K/Akt pathway in EOC cells plays an important role in the resistance to TRAIL-induced apoptosis.

The objectives of my project were to demonstrate that Akt is implicated in the regulation of TRAIL-induced apoptosis in EOC cells and to investigate the mechanisms by which Akt contributes to TRAIL resistance.

We report that Akt activation reduces the sensitivity of ovarian cancer cells to TRAIL. TRAIL-resistant cells were sensitized to TRAIL-induced apoptosis by treatment with PI3K or Akt inhibitors but inhibition of PI3K/Akt signaling pathway did not interfere with the recruitment and processing of the pro-caspase-8 to the death-inducing signaling complex (DISC). Conversely, overexpression of Akt1 in TRAIL-sensitive cells promoted resistance to TRAIL. Despite the fact that TRAIL-induced caspase-8 activation was observed in both TRAIL-sensitive and –resistant cell lines, Bid cleavage occurred only in TRAIL-sensitive cells. Akt activation in TRAIL-sensitive cells inhibited TRAIL-induced Bid cleavage. Furthermore, Bid expression was downregulated by Akt activation. Depletion of Bid by siRNA in TRAIL-sensitive EOC cells was associated with a decrease in TRAIL-mediated apoptosis and Bid overexpression in TRAIL-resistant cells resulted in increased TRAIL-mediated apoptosis.

Altogether, these results suggest that Akt is a critical factor for mediating intrinsic TRAIL resistance among EOC cells and that an important mechanism by which Akt activation contributes to TRAIL resistance is by regulating the expression of pro-apoptotic protein Bid.

Given these data, we speculate that Akt activation may be a potential biomarker to predict patient's response to TRAIL therapy and that the inhibition of the PI3K/Akt pathway can become one of the strategies to overcome resistance to TRAIL therapy in ovarian cancer.

Key words: epithelial ovarian cancer; TRAIL; apoptosis; resistance; Bid.

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LIST OF ABBREVIATIONS

A1/Bfl-1	Bcl-2 related protein A1
ABX-EGF	Human monoclonal antibody specific to the epidermal growth factor receptor
APAF1	Apoptotic protease activating factor-1
APST	Atypical proliferative serous tumor
ASK 1	Apoptosis signal-regulating kinase 1
ATCC	American Type Culture Collection
ATP	Adenosine-5'-triphosphate
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
Bid	BH3 interacting domain death agonist
Bik	Bcl-2-interacting killer
Bim	Bcl-2 interacting protein
Bmf	Bcl-2-modifying factor
BNIP3L	Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3-like
Bok	Bcl-2-related ovarian killer protein
Boo/Diva	Divergent Bcl-2 protein
bp	Base pair(s)
BRAF	B-Raf proto-oncogene serine/threonine-protein kinase
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BSO	Bilateral salpingo-oophorectomy
CA 125	Cancer antigen 125 or carbohydrate antigen 125
CAMs	Cell adhesion molecules
c-FLIP$_L$	FLICE-inhibitory protein long form
c-FLIPs	FLICE-inhibitory protein short form
CIC	Cortical inclusion cysts
COX IV	Enzyme cytochrome c oxidase or Complex IV
CREB	Cyclic AMP-response element-binding protein
CTL	Cytotoxic T lymphocytes
DcR1	Decoy receptor 1
DcR2	Decoy receptor 2
DD	Death domain
DED	Death effector domain
DISC	Death inducing signaling complex
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DNA	Deoxyribonucleic acid
DR4	Death receptor 4
DR5	Death receptor 5
ECM	Extracellular matrix

EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EOC	Epithelial ovarian cancer
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
EV	Empty vector
FADD	Fas-associated protein with death domain
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FOXO	Forkhead box proteins
FSH	Follicle-stimulating hormone
FTE	Fallopian tube epithelium
G1 phase	Growth phase of cell cycle
GF	Growth factor
GFP	Green fluorescent protein
GPCR	G-protein-coupled receptor
GPI	Glycosylphosphatidylinositol
GSK-3	Glycogen synthase kinase 3
HE4	Human epididymis protein 4
HeLa	Immortal cell line
Her2/neu	Human Epidermal growth factor Receptor 2
Hrk	Activator of apoptosis harakiri
IAP	Inhibitor of apoptosis proteins
IC_{50}	Half maximal inhibitory concentration
IkB	Inhibitor of NF-kappaB kinase
IKK	IkappaB kinase
IL	Interleukin
JNK	C-Jun N-terminal kinase
Kb	Kilo base pairs
kDa	kilodaltons
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LH	Luteinizing hormone
LPA	Lysophosphatidic acid
mAbs	Monoclonal antibodies
MAPK	Mitogen-activated protein (MAP) kinases
Mcl-1	Myeloid Cell Leukemia 1
Mdm2	Murine double minute 2
MKK4	Mitogen-activated protein kinase kinase 4
MLK3	Mixed lineage kinase 3
MMP	Matrix metalloproteinase
MMP	Mitochondrial membrane permeabilization
MPST	Micropapillary serous carcinoma
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NCCN	National Comprehensive Cancer Network
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NK	Natural killer
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
OCP	Oral contraceptive pill
OPG	Osteoprotegerin
OSE	Ovarian surface epithelium
P21/WAF	Cyclin-dependent kinase inhibitor 1
P27/Cip/Kip	Cyclin-dependent kinase inhibitor 1B
P38	P38 mitogen-activated protein kinases
pAkt	Phosphorylated Akt
PCOS	Polycystic ovary syndrome
PDGFR	Platelet-derived growth factor receptor
PDK-1	Protein serine/threonin kinase 3-phosphoinositide-dependent kinase-1
PH	Pleckstrin-homology domain
PI3K	Phospatidylinositol-3 kinase
PIP2	Phospatidylinositol (4, 5)-biphosphate
PIP3	Phospatidylinositol (3, 4, 5)-trisphosphate
PKA	Protein kinase A
PKB	Protein kinase B (Akt)
PKC	Protein kinase C
pRb	Retinoblastoma protein
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PUMA	P53 upregulated modulator of apoptosis
qRT-PCR	Quantitative real time polymerase chain reaction
Raf	Proto-oncogene serine/threonine-protein kinase
Ras	Protein superfamily of small GTPases
RIP	Receptor interacting protein 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPTK	Receptors with protein tyrosine kinase activity
S phase	Synthesis phase
SAPK	Stress-activated protein kinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
siRNA	Small interfering RNA
Smac/Diablo	Second mitochondria-derived activator of caspase/direct IAP binding protein with low pI
TAMs	Tumor-associated macrophages
tBid	Truncated BH3 interacting domain death agonist
Thr	Threonine
TIC	Tubal intraepithelial carcinoma
TM	Transmembrane
TNF	Tumor necrosis factor
TP53	Tumor protein 53 gene
TRAF-2	TNF receptor associated protein
TRAIL	Tumor necrosis factor (TNF)-related apoptosis inducing ligand
TRAIL-R	Tumor necrosis factor (TNF)-related apoptosis inducing ligand receptor
TSC	Tuberous sclerosis complex

TVS	Transvaginal sonography
VEGF	Vascular endothelial growth factor
WHO	World health organization
WT	Wild type
XIAP	X-linked inhibitor of apoptosis protein

INTRODUCTION

1. Cancer

1.1 General remarks

Cancer is a genetic disease that is responsible for one in eight deaths worldwide (GARCIA *et al.,* 2007). The two main characteristics of the cancer are the uncontrolled growth of the cells in the human body and the ability of these cells to migrate from the original site and spread to distant sites. Cancer is a leading cause of death in Canada. Based on the 2009 incidence rates, 40% of Canadian women and 45% of men will eventually develop cancer. An estimated 1 out of every 4 Canadians are expected to die from cancer. The most common types of cancer are lung, breast and prostate cancer (CANADIAN CANCER STATISTICS, 2009).

1.2 The hallmarks of cancer

Development of cancer involves dynamic changes in the genome. In order to be able to transform from normal to malignant, cells can accumulate many genetic alterations during lifetime, which explains the dramatic age-dependent escalation in cancer risk. Cancer cells acquire a common set of properties such as self-sufficiency in growth signals, evasion of programmed cell death (apoptosis), insensitivity to growth-inhibitory signals, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. These new properties are shared by most types of human tumors (HANAHAN and WEINBERG, 2000).

Multiple systems in the cells ensure that the mutations are rare events. So rare that the multiple mutations known to be present in tumor cell genomes are highly unlikely to occur during human lifetime. Yet cancers do appear because genomes of tumor cells have increased mutability due to malfunction of specific components of genomic caretaker

systems. Not surprisingly, in addition to six classical hallmarks of cancer mentioned above, a set of additional hallmarks were proposed: DNA damage stress, proteotoxic stress, mitotic stress, metabolic stress, oxidative stress and evading immune surveillance (LUO *et al.*, 2009) (Figure 1).

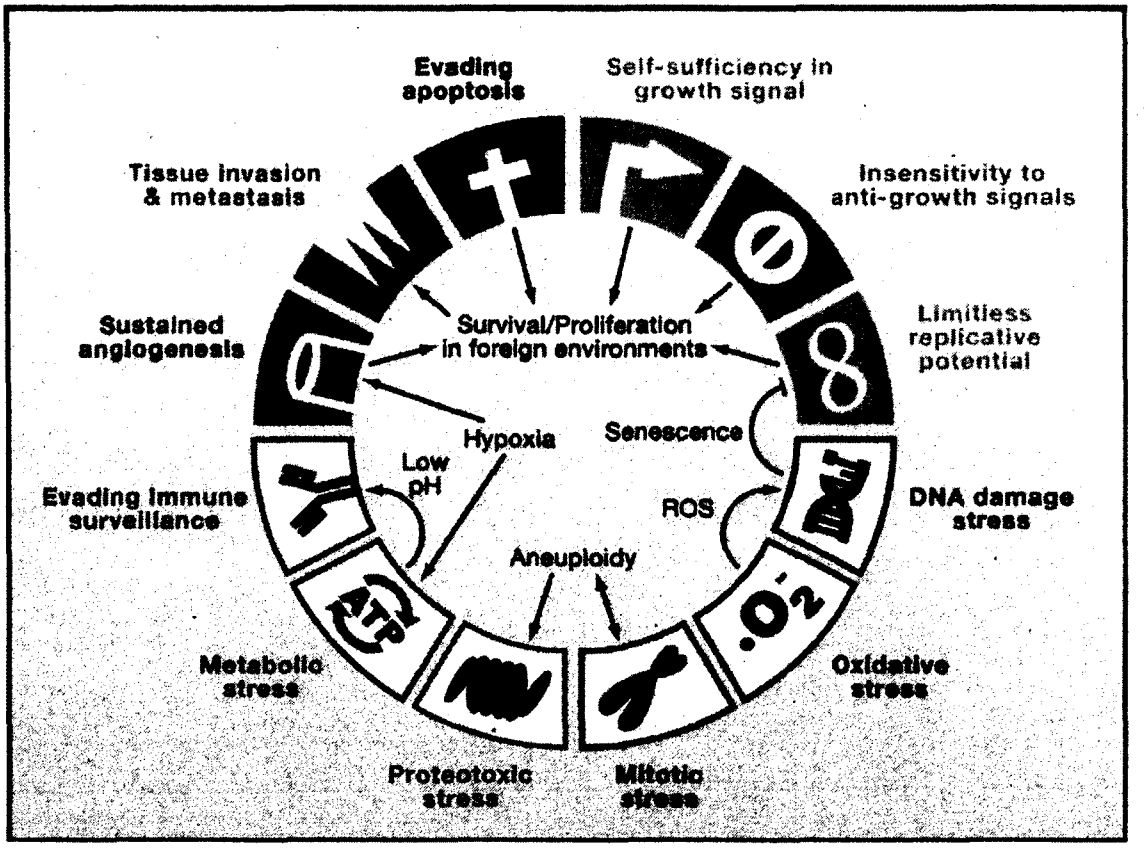

Figure 1. The hallmarks of cancer (LUO *et al.*, 2009). In addition to the six hallmarks of cancer originally proposed by Hanahan and Weinberg (top half, white symbols) and evasion of immune surveillance proposed by Kroemer and Pouyssegur, a set of additional hallmarks were highlighted (lower half, colored symbols).

1.2.1 Self-sufficiency in growth signals

Normal cells require mitogenic growth signals to proliferate. These signals are transmitted into the cells by transmembrane receptors that bind number of signaling molecules: GFs (growth factors), components of ECM (extracellular matrix), and cell-to-cell adhesion/interaction molecules. Normal cells can not proliferate in absence of such stimulatory signals. Tumor cells generate many of their own GFs, thereby reducing their dependence on stimulation from their normal tissue microenvironment (FEDI *et al.*, 1997).

Cancer cells can achieve independence from exogenously derived GFs by three common molecular strategies, involving alterations of extracellular growth signals, or transcellular transducers of those signals, or intracellular molecules that translate those signals into action.

Successful tumor cells can influence their normal neighbours by inducing them to release growth-stimulating factors (SKOBE and FUSENIG, 1998). In addition, the inflammatory cells attracted to sites of neoplasia may promote (rather than eliminate) cancer cells (HUDSON *et al.*, 1999; COUSSENS *et al.*, 1999). Tumor cells produce various cytokines and chemokines that attract the diverse leukocyte population — for example, neutrophils, dendritic cells, macrophages, eosinophils and mast cells, as well as lymphocytes — all of which are capable of producing an assorted array of cytokines, cytotoxic mediators including reactive oxygen species, serine and cysteine proteases, MMPs and membrane-perforating agents, and soluble mediators of cell killing, such as TNF-α, interleukins and interferons (KUPER *et al.*, 2000). But the metabolic microenvironment of tumor cells may inhibit the function of antitumor immune effectors such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells while attracting inflammatory cells that participate in tumor progression such as tumor-associated macrophages (TAMs) (WAHL and KLEINMAN, 1998). TAMs and tumor cells produce IL-10, which effectively blunts the anti-tumour response by cytotoxic T cells (KROEMER and POUYSSEGUR, 2008). TAMs facilitate angiogenesis, promote tumor cell migration, and exert local immunosuppressive effects

(CONDEELIS and POLLARD, 2006). In addition, acidification of tumor beds can inhibit the activity of NK cells (LARDNER *et al.*, 2001).

The cell surface receptors that transduce growth-stimulatory signals into the cell are often overexpressed in cancers. Cancer cells can also switch the types of the extracellular receptors (integrins) they express, favouring the ones that transmit pro-growth signals (GIANCOTTI and RUOSLAHTI, 1999).

A number of intracellular growth signaling pathways are deregulated in cancer cells. One of them is SOS-Ras-Raf-MAP kinase mitogenic cascade (HUNTER, 1997) which is linked to a variety of cross-talking pathways. For example, the direct interaction of Ras protein with the survival-promoting PI3K/Akt pathway enables GFs to stimulate survival signals within the cell (DOWNWARD, 1998).

1.2.2 Insensitivity to antigrowth signals

Within a normal tissue, multiple antiproliferative signals can block proliferation by two distinct mechanisms. Cells may be forced out of proliferative cycle into the quiescent (G0) state from which they can recover upon GFs stimulation. Alternatively, cells may be induced to permanently exit their proliferative state by entering into a postmitotic state. For example, when they are terminally differentiated, cells permanently enter G0 phase but continue to perform their main functions for the rest of the organism's life (HANAHAN and WEINBERG, 2000).

Tumor cells use various strategies to avoid terminal differentiation. The classical example is negative regulation of pRb tumor suppressor gene which normally blocks proliferation by altering function of E2F transcription factor that is essential for progression from G1 to S phase (WEINBERG, 1995). Another strategy is overexpression of the c-Myc oncoprotein that drives cell growth and proliferation (FOLEY and EISENMAN, 1999).

1.2.3 Resistance to apoptosis

The apoptotic program is present in latent form in almost all cell types. Intracellular sensors monitor the cell's well being and activate the death pathway in response to detected diverse abnormalities such as DNA damage, signaling imbalance provoked by oncogene action, survival factors insufficiency, or hypoxia (EVAN and LITTLEWOOD, 1998).

Resistance to apoptosis is acquired by cancer cells through occurring loss of pro-apoptotic regulators such as p53 tumor suppressor gene that is seen in more than 50% of human cancers (HARRIS, 1996). Additionally, constitutive activation of the PI3K/Akt pro-survival pathway that counteracts apoptosis is often observed in cancer cells (EVAN and LITTLEWOOD, 1998; DOWNWARD, 1998; CANTLEY and NEEL, 1999). Possible mechanisms of action are described in section 4.3.1 and 4.3.2.

1.2.4 Limitless replicative potential

Once normal cell populations have progressed through a certain number of doublings, they stop growing (a process termed senescence). This process in independent of cell-to-cell signaling pathways described above. The number of cell divisions is controlled by the end of the chromosomes (telomeres). Telomeres keep chromosomes protected and prevent them from fusing into rings or binding with other DNA. After each cell replication 50 to 100 bp of telomeric DNA are lost. As human telomeres grow shorter, eventually cells reach the limit of their replicative capacity and progress into senescence. However, further cell proliferation can be achieved by inactivation of p53 and pRb pathways. Cells entering proliferation after inactivation of p53 and pRb pathways undergo crisis. Crisis is characterized by gross chromosomal rearrangements and genome instability, and almost all cells die (COUNTER *et al.*, 1992). But 85-90% of tumor cells are able to maintain telomere length by upregulating expression of the enzyme *telomerase* that adds hexanucleotide repeats at the end of telomeric DNA, which in turn permits unlimited multiplication of cells (BRYAN and CECH, 1999).

1.2.5 Sustained angiogenesis

The nutrients and oxygen supply by the vasculature are crucial for cell function and survival. The process of new blood vessels formation (angiogenesis) is carefully regulated in normal tissues. Counterbalancing positive and negative signals encourage or block angiogenesis. Some examples of angiogenesis-initiating factors are VEGF (vascular endothelial growth factor) and FGF1/2 (basic fibroblast growth factors) (VEIKKOLA and ALITALO, 1999) and one of the inhibitors is thrombospodin-1 (BULL *et al.*, 1994). Tumors appear to activate the angiogenic switch by changing the balance of angiogenesis inducers and countervailing inhibitors (HANAHAN and FOLKMAN, 1996).

1.2.6 Tissue invasion and metastasis

The distant settlements of tumor cells (metastases) are the cause of 90% of human cancer deaths (SPORN, 1996). The capability for invasion and metastasis enables cancer cells to escape the primary tumor mass and form new tumors in other parts of the body where, at least initially, nutrients and space are not limiting. Several classes of proteins are involved in this process: the cell-cell adhesion molecules (CAMs), the integrins, which link cells to extracellular matrix, the cadherins and extracellular proteases (WOODHOUSE *et al.*, 1997).

The most widely observed alteration in cell-to-environment interactions in cancer involves E-cadherin, a homotypic cell-to-cell interaction molecule expressed on cell surface of epithelial cells. E-cadherin function is apparently lost in a majority of epithelial cancers (CHRISTOFORI and SEMB, 1999) with the exception of epithelial ovarian cancer. Up-regulation of E-cadherin is an early defining event in ovarian cancer and may play a significant role in the initial development of the primary ovarian tumor (ANSENBERGER *et al.*, 2009).

Another general parameter of the invasive metastatic capability involves extracellular proteases (COUSSENS and WERB, 1996). Protease genes are upregulated, protease inhibitor genes are downregulated, and inactive zymogen forms of proteases are converted into active enzymes in cancer cells (STETLER-STEVENSON, 1999).

1.2.7 Additional hallmarks: the stress phenotypes of cancer

DNA damage stress: Tumors pass through stages of extreme genomic instability that result in accumulation of point mutations, deletions, complex chromosomal rearrangements, and extensive aneuploidy (HARTWELL and KASTAN, 1994). This is due to a constitutive level of endogenous DNA damage, which leads to activation of the DNA damage checkpoint and thereby to apoptosis or cell cycle arrest. Cancer cells are able to overcome the antiproliferative effects of DNA damage, continuing to replicate in the presence of damage.

Proteotoxic stress: The long-term health of the cell has been linked to protein quality control. Under optimal conditions this is accomplished by protein homeostasis, a highly complex network of molecular interactions that balances protein biosynthesis, folding, translocation, assembly/disassembly, and clearance. If unattended, misfolded proteins can result in severe molecular damage to the cell. Adaptation and survival requires the ability to sense damaged proteins and to coordinate the activities of proteotoxic stress response pathways and chaperone networks. Yet, despite the abundance and apparent capacity of chaperones and other components of homeostasis to restore folding equilibrium, cancer cells have been shown to contain increased amount of toxic, unfolded protein aggregates (DENOYELLE *et al.*, 2006).

Mitotic stress: The completion of spindle formation is a crucial transition point in the cell cycle called *the spindle assembly checkpoint*. If some chromosomes are not properly attached to the mitotic spindle by the time of this checkpoint, the onset of anaphase will be delayed. A subset of tumors have increased defects in mitotic proteins that execute chromosome segregation and defects in the spindle assembly checkpoint, which

coordinates anaphase entry with the proper alignment of chromosomes on the mitotic spindle (CAHILL *et al.*, 1998).

Metabolic stress: Normal cells carry out mitochondrial oxidative phosphorylation to produce ATP. Most cancer cells produce energy by less efficient method of glycolysis and secrete a large amount of lactic acid, even under high oxygen conditions (DEBERARDINIS *et al.*, 2007) which provides several advantages to the tumor including adaptation to a low oxygen environment and acidification of the surrounding microenvironment, which promotes tumor invasion and suppresses immune surveillance (GATENBY and GILLIES, 2004).

Evading immunosurveillance: The metabolic microenvironment of tumor cells may inhibit the function of antitumor immune effectors such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells while attracting inflammatory cells that participate in tumor progression. Tumor-associated macrophages (TAMs) are often enriched in hypoxic and tumor perinecrotic areas and constitute a negative prognostic marker. TAMs facilitate angiogenesis, promote tumor cell migration, and exert local immunosuppressive effects (CONDEELIS and POLLARD, 2006).

2. Ovarian cancer

2.1 General remarks

Ovarian cancer occurs in approximately 204 000 women worldwide each year (JEMAL *et al.*, 2008). Globally it claims 125 000 lives per year, making it the seventh leading cause of cancer-related deaths among women. Over 2500 Canadian women are diagnosed every year; and every year 1700 women succumb to this disease (CANADIAN CANCER STATISTICS, 2009). Despite its relatively low incidence rate, ovarian cancer is an extremely lethal disease. Its high mortality is mostly due to the difficulty in diagnosing the disease at early stage. Although the five years survival rate for stage I ovarian cancer is

>90%, stage I diagnoses are more often the exception than the rule. Most patients (75%) present with advanced stage (III/IV) tumors, for which the five year survival rate is at best 30% (BOYLE and LEVIN, 2008). Consequently, ovarian cancer is frequently nicknamed the "silent killer".

2.2 Risk factors

Age: Epithelial ovarian cancer (EOC) is a disease of older age. With the exception of the hereditary forms of the disease, ovarian cancer is uncommon before the age of 40 years (COLOMBO *et al.*, 2006).

Environmental and hormonal factors: Significant geographic and ethnic variations in ovarian cancer incidence have been observed. Rates are highest for Caucasian women in industrialized countries of North America and Europe which could be explained by differences in reproductive pattern and diet. Epidemiological research has shown that multiple pregnancies, lactation, and oral contraceptive use are associated with a reduced risk of developing ovarian cancer (TORTOLERO-LUNA and MITCHEL, 1995). Conversely, nulliparity, early menarche, and late menopause are associated with an increased risk (OZOLS *et al.*, 2004). Studies that analyzed dietary factors found that diet, which is high in meats and low in vegetables, may be positively associated with ovarian cancer incidence. Vegetable, but not fruit, consumption may be associated with beneficial effects (SCHULZ *et al.*, 2004). Exposure to talc or asbestos may represent factors that may initiate ovarian cancerogenesis, inducing a chronic inflammation of ovarian epithelium (COLOMBO *et al.*, 2006).

Hereditary predisposition: A strong family history of either breast or ovarian cancer is the most important risk factor for the development of EOC. Approximately 10% to 15% of all EOC have a hereditary predisposition (PAL *et al.*, 2005). Hereditary ovarian cancer is seen commonly within the breast-ovarian cancer family syndrome because of mutations in BRCA1or BRCA2 (GARBER and OFFIT, 2005). BRCA1 and BRCA2 genes are located on chromosomes 17q and 13q respectively. The proteins encoded by these genes are

implicated in DNA repair (SCULLY *et al.*, 1996). Ovarian cancer has also been seen in families with hereditary non-polyposis colon cancer (Lynch syndrome II), along with an excess of colorectal and endometrial cancers. Lynch syndrome II results from inherited mutation in DNA mismatch repair genes (LINDOR *et al.*, 2006).

2.3 Diagnostic cues

Symptoms: Ovarian carcinoma does not produce specific symptoms. Abdominal discomfort or vague pain, abdominal fullness, bowel habit changes, constipation, early satiety, dyspepsia, and bloating, urinary frequency are frequent presenting symptoms. Often these symptoms occur when the disease is already spread throughout the abdominal cavity (ALETTI *et al.*, 2007).

Signs: The most important sign of ovarian carcinoma is the presence of a pelvic mass on physical examination. The ovaries are a pair of tiny organs, only 2-4 cm in diameter, suspended on either side of uterus and not readily accessible by pelvic examination unless significantly enlarged (Figure 2). By definition, a stage I tumor is confined to the ovary and is unlikely to be noticed without the aid of a sensitive screening test. The best tools currently available are transvaginal sonography (TVS) and serum biomarker testing (KARST and DRAPKIN, 2010).

2.3.1 Serum biomarkers

The most well-studied ovarian cancer biomarker is CA-125, a high molecular weight transmembrane glycoprotein expressed by coelomic- and Müllerian-derived epithelia. It is not expressed by normal ovarian epithelium (KABAWAT *et al.*, 1983). CA-125 is detected at low levels (<35 U/ml) in the serum of healthy individuals but is elevated in 50% of stage I ovarian cancer patients and 90% of advanced-stage patients (BAST *et al.*, 2002). However, it was later discovered that serum CA-125 can also be increased in different benign conditions (such as pelvic inflammatory disease, endometriosis, uterine fibroids, and ovarian cysts) making false positivity a problem (MEDEN and FATTANI-MEIODI,

1998). In addition, 25% of ovarian carcinomas are CA-125 negative (MANN *et al.*, 1988). Although, CA-125 has not demonstrated adequate sensitivity to support its use in screening for early-stage ovarian cancer, it remains a valuable tool for monitoring response to chemotherapy and for detecting disease relapse following treatment (GADDUCCI *et al.*, 1995). Recent studies identified several new candidates for markers such as LPA (lysophosphatidic acid) that is found to be elevated in serum and ascites fluid (XU *et al.*, 1998), mesothelin (SCHOLLER *et al.*, 1999), HE4 (SCHUMMER *et al.*, 1999), osteopontin (MCINTOSH *et al.*, 2004), VEGF and IL8 (LU et *al.*, 2004) and macrophage colony-stimulating factor (XU *et al.*, 1991).

2.4 Grading of ovarian cancer

90% of ovarian cancers arise from cells that make up the epithelial layer that covers the surface of the ovaries. Other two types are germ cell tumors and stromal tumors. Stromal tumors arise in hormonally active elements within the connective tissue stroma of the ovary. Germ cell tumors and stromal tumors each account for approximately 5% of ovarian cancers (GERSHENSON *et al.*, 2005). Epithelial ovarian cancer (EOC) represents a heterogeneous group of neoplasm that exhibit a wide range of tumor morphologies, clinical manifestations, and underlying genetic alterations.

Ovarian tumors are surgically staged to determine how far they have extended beyond ovary. Stage I indicates confinement to ovary. Stage II tumors extended beyond ovary to adjacent pelvic structures such as fallopian tube or uterus. Stage III indicates metastasis to the peritoneum and/or regional lymph nodes. Stage IV tumors have metastasized beyond the peritoneum to distant sites.

World health organization (WHO) recognizes eight histologic tumor subtypes of EOC: serous, endometrioid, mucinous, clear cell, transitional cell, squamous cell, mixed epithelial, and undifferentiated (TAVASSOLI and DEVILEE, 2003). The three most common (serous, endometrioid, and mucinous) are characterized by their morphological resemblance to various mucosal tissues of female reproductive tract, all of them exhibit

Müllerian differentiation (Figure 2). It has been estimated that 50% of malignant ovarian tumors are serous carcinomas, while 25% are endometrioid, 10% are mucinous and only 5% are clear cell carcinomas (CHEN *et al.*, 2003).

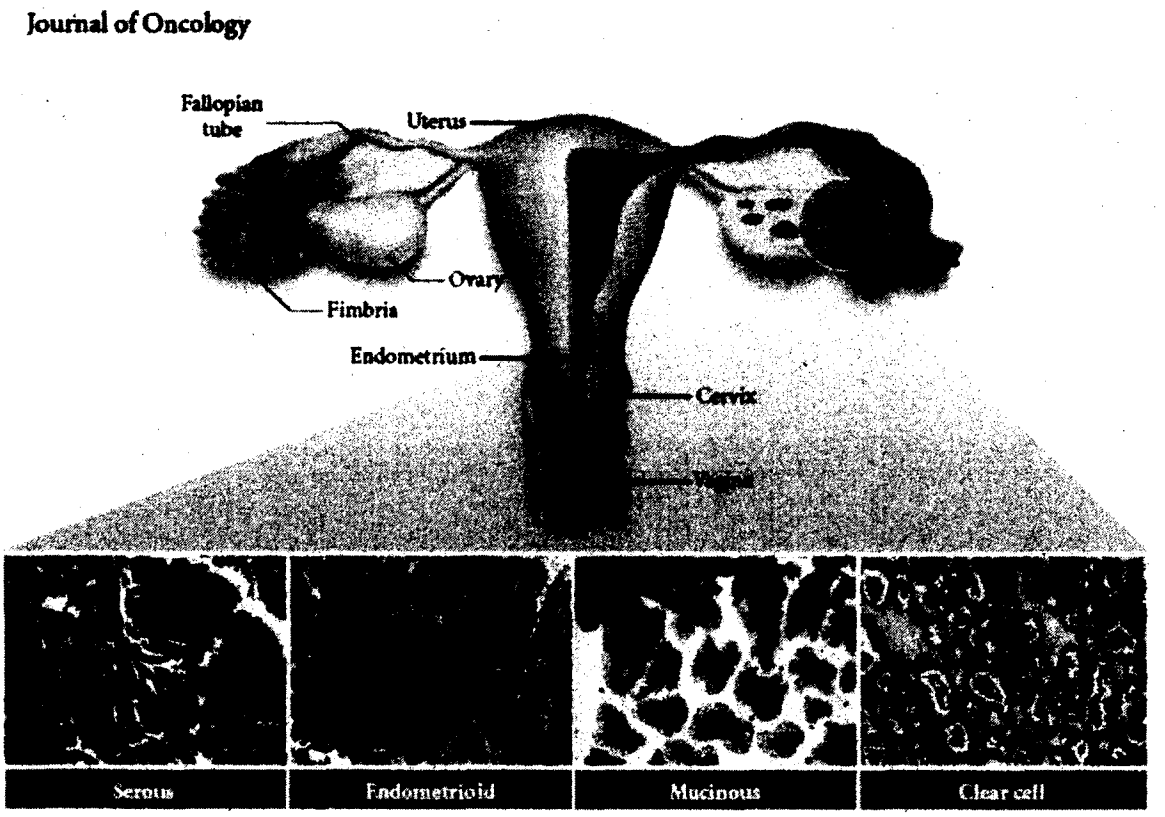

Figure 2. The major histologic subtypes of ovarian carcinoma (KARST and DRAPKIN, 2010). Serous carcinomas resemble fallopian tube epithelium, endometrioid carcinomas resemble endometrial glands, and mucinous carcinomas resemble endocervical epithelium. Photographs show representative tumor sections stained with hematoxylin and eosin. The shaded circle represents the general anatomical location from which ovarian carcinomas are thought to arise. The pink and blue entities within the cross-sected ovary represent maturing ovarian follicles.

Within each subtype, tumors are further described as *benign* (localized tumors that has not spread to other parts of the body), *malignant* (tumors that invade and damage other tissues and organs), or *borderline* (semimalignant tumors which are considered to have low malignant potential) and, depending upon tumor subtype, classified as low-or high-grade. *Low grade tumors* are composed of uniform cells that look very like normal cells of ovary with mild or moderate nuclear atypia and low mitotic index. They are associated with a serous neoplasm of low malignant potential. *High grade tumors* are composed of pleomorphic cells that look very abnormal with marked nuclear atypia and high mitotic index and associated with high malignant potential (MALPICA, 2008).

2.5 Role of EOC ascites in promotion of tumor cell survival

Epithelial ovarian cancer (EOC) is highly metastatic cancer characterized by widespread intraperitoneal dissemination and ascites formation (an abnormal accumulation of fluid in the abdomen) (SHEN-GUNTHER and MANNEL, 2002). EOC ascites may contain a variety of cytokines, growth factors, bioactive lipids, hormones, and components of ECM which contribute to cancer cell migration and invasion (YAMADA *et al.*, 2004; GRAVES *et al.*, 2004). Our laboratory has demonstrated that EOC ascites contribute to tumor cell survival by activating the pro-survival PI3K/Akt pathway (LANE *et al.*, 2007; LANE *et al.*, 2010). Activation of a number of cell surface receptors may contribute to the activation of PI3K/Akt pathway (Figure 3).

Ovarian cancer ascites contains growth factors that could potentially activate tyrosine kinase receptors (ABENDSTEIN *et al.*, 2000; MIYAMOTO *et al.*, 2004). The binding of growth factors to tyrosine kinase receptors stimulates the phosphorylation of phosphatidylinositol 3-kinase (PI3K), which in turn leads to Akt activation (Figure 3). Epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and Her2/neu are commonly overexpressed in EOC and are involved in inducing the tumor cell proliferation (ALPER *et al.*, 2001; MATEI *et al.*, 2006).

PI3K may also become activated through G-protein-coupled receptors (GPCR) (Figure 3). G-protein-coupled receptors regulate a variety of cell functions including cell proliferation, survival, and motility, and have recently emerged as important players in tumor growth and survival (MILLS and MOOLENAAR, 2003). One of the ligands of G-protein coupled receptors is the lysophosphatidic acid (LPA) that has been shown to induce cell survival signaling through the PI3K/Akt pathway in ovarian cancer cells (KANG *et al.*, 2004). Ascites fluid contains significant levels of LPA, which exceed levels required to activate LPA receptors (YAMADA *et al.*, 2004).

PI3K/Akt pathway may also become activated by the interactions between extracellular matrix proteins (ECM) with cell surface integrins (HYNES, 1992). Integrins transmit signals directly through ligation-dependent recruitment of the focal adhesion kinase (FAK) leading to the activation of several cell-signaling pathways including the PI3K/Akt pathway (STUPACK and CHERESH, 2002).

Our laboratory investigated the mechanisms by which EOC ascites activates Akt in EOC cells and we demonstrated that survival promoting activity of ascites was not affected by inhibitors of GF receptors including EGFR, VEGFR, FGFR, Her2/neu, and IGF-R1. We also concluded that LPA does not contribute to the pro-survival activity of EOC ascites. However, the integrin pathway was involved in ascites-mediated protection from TRAIL-induced apoptosis. We demonstrated that ovarian cancer ascites induces FAK and Akt activation in an $\alpha v\beta 5$ integrin-dependent pathway (LANE *et al.*, 2010).

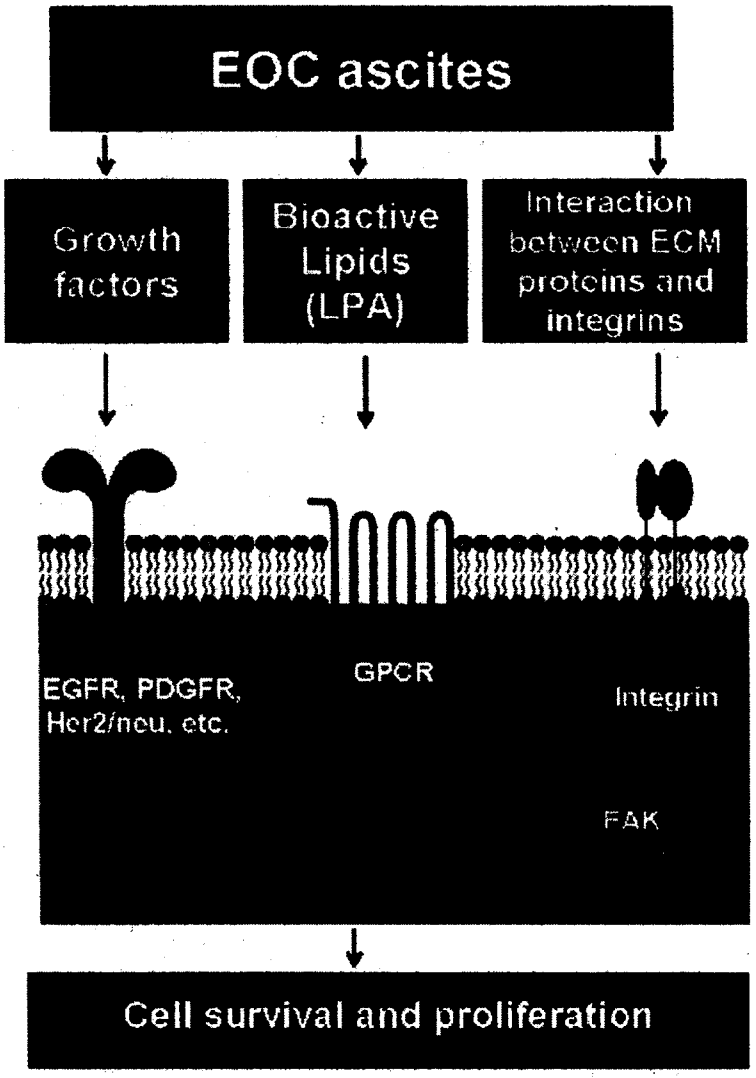

Figure 3. The mechanisms of PI3K/Akt pathway activation by ascites. Ovarian cancer ascites contains a number of growth factors that could potentially activate tyrosine kinase receptors such as EGFR, PDGFR, Her2/neu, etc. Ascites fluid also contains bioactive lipids, such as LPA, which is one of the ligands of G-protein coupled receptors (GPCR). Activation of these receptors can result in downstream activation of PI3K/Akt. Akt pathway can also become activated due to the interactions between extracellular matrix (ECM) proteins with cell surface integrins. Such interactions activate the focal adhesion kinase (FAK) and subsequently the PI3K/Akt pathway.

2.6 Origin of ovarian cancer

Several hypotheses have been proposed about the underlying physiological processes that increase risk of the ovarian cancer (Table 1) (LANDEN *et al.*, 2008).

Table 1. Hypotheses on physiologic susceptibilities to epithelial ovarian cancer

Hypothesis	Proposed mechanisms	Best evidence
Gonadotropin stimulation	Stimulatory effect of FSH and LH promote growth, increased cell divisions, and mutations	Increased EOC risk with infertility, PCOS; Decreased risk with progesteron-only OCPs; FSH upregulates many oncogenes and promotes growth in preclinical models
Inflammation	Damaged OSE with ovulation induces inflammation, which promotes reconstruction and mutation susceptibility	Possible reduced risk with NSAID use; increased risk with talc or asbestos; abundance of inflammatory mediators in tumors

Abbreviations: OSE, ovarian surface epithelium; EOC, epithelial ovarian cancer; OCP, oral contraceptive pill; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PCOS, polycystic ovarian syndrome

2.6.1 Ovarian surface epithelium and cortical inclusion cyst

It is widely thought that most ovarian cancers develop from the surface epithelium (OSE). OSE is a flat-to-cuboidal layer of uncommitted mesothelial cells covering the exterior surface of the ovary. During ovulation, follicular rupture and oocyte release causes physical trauma upon the ovarian surface, creating a breach in OSE that must be repaired. Over the course of a woman's life, this process of damage and repair is repeated multiple times. Accordingly OSE exhibit a high degree of plasticity that facilitates tissue remodeling (KRUK *et al.*, 1994). In addition to physical trauma, OSE cells are subjected to ovulation-associated inflammatory cytokines and oxygen species that are capable damaging DNA

(MURDOCH and MARTINCHICK, 2004). Furthermore, as women age, the ovarian surface develops numerous invaginations into the cortical stroma. These invaginations frequently pinch off and become entrapped within the stroma, forming circular OSE-lined structure termed "cortical inclusion cysts" (CICs) (Figure 4). Once inside the ovary, the epithelial cells lining CICs are exposed to a new hormone-rich milieu that is thought to induce a differentiation into more complex epithelium resembling that of Müllerian-derived organs (DRAPKIN and HECHT, 2006). If the epithelial cells also happen to harbour unresolved DNA damage, they may be prime targets for neoplastic transformation, eventually giving rise to ovarian carcinomas.

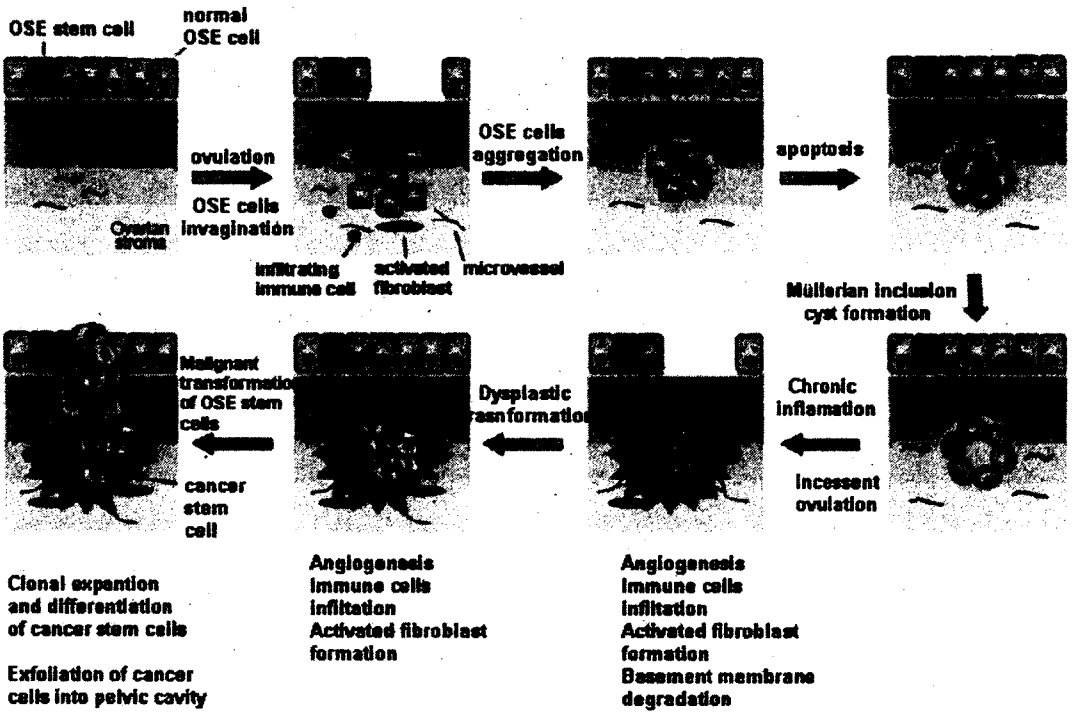

Figure 4. Model of the development of an EOC tumor (FARLEY *et al.*, 2008). Incessant ovulation and wound repair increases the risk of genetic abnormalities, leading to changes in epithelial cells lining the Müllerian inclusion cyst. Stromal microenvironment in the forms of activated fibroblast formation, microvessel proliferation, and growth factors contributes to dynamic formation and eventual malignant transformation.

2.6.2 Two pathway model of ovarian carcinogenesis

The two pathway model of ovarian carcinogenesis proposed by KURMAN and SHIH (Figure 5) takes into account the diverse nature of ovarian cancer and correlates the clinical, pathological, and molecular features of the disease. In this model, ovarian tumors are divided into type I and II. Type I tumors are slow growing, generally confined to the ovary at the diagnosis, and develop from well-established precursor lesions. Type I tumors include low-grade micropapillary serous carcinoma, mucinous, endometrioid, and clear cell carcinomas. They are genetically stable and characterized by mutations in number of different genes including KRAS, BRAF, PTEN, and β-catenin. Mutations of KRAS and BRAF lead to the activation of MAPK signaling pathway (PEYSSONNAUX and EYCHENE, 1996). PTEN mutations result in constitutively PI3K/Akt signaling (OBATA *et al.*, 1998).

Type II tumors are rapidly growing highly aggressive neoplasms for which well-defined precursor lesions have not been described. Type II tumors include high-grade serous carcinoma, malignant mixed mesodermal tumors, and undifferentiated carcinomas. This group has a high level of genetic instability and is characterized by mutation of TP53. These tumors may also exhibit gene amplification and overexpression of HER2/neu oncogene (10-20%) (ROSS *et al.*, 1999) and Akt2 oncogene (12-18%) (CHENG *et al.*, 1992) and they are rarely only confined to the ovary.

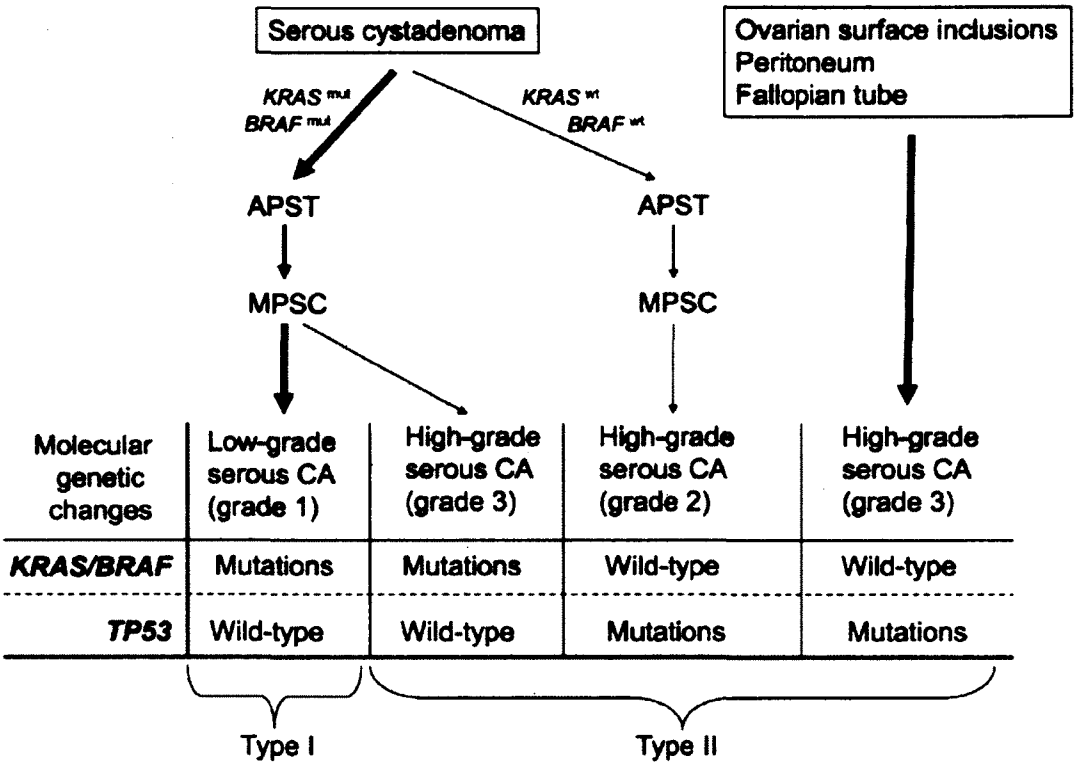

Figure 5. Two pathway model of ovarian carcinogenesis (KURMAN and SHIH, 2008). Proposed pathways for the development of different types of serous carcinoma. APST indicates atypical proliferative serous tumor; MPSC, micropapillary serous carcinoma.

2.6.3 Fallopian tube as a site of origin

Several recent studies have demonstrated that many high-grade serous ovarian tumors may actually be of tubal origin, arising from the distal region of the fallopian tube, but then quickly spreading to nearby ovary (MEDEIROS *et al.*, 2006; KINDELBERGER *et al.*, 2007; LEE *et al.*, 2007). The fimbria (the end of the Fallopian tube (Figure 2)) lies in extremely close proximity to ovarian surface epithelium and is therefore exposed to the same inflammatory and potentially carcinogenic microenvironment. Lee *et al.* formulated a model of ovarian cancer which incorporates the fimbria as a major site of origin for serous carcinomas. This model states that there are two distinct pathways leading to ovarian tumorigenesis. The first route is the traditional OSE-CIC pathway which leads to formation of Kurman's and Shin's "Type I" tumors. The second pathway involves the fallopian tube fimbria, where a combination of TP53 mutation and genotoxic stress leads to the clonal expansion of secretory epithelial cells, forming a pre-neoplastic precursor lesion or "p53 signature". Additional genetic "hits" in the absence of functional TP53 enable these cells to acquire a proliferative capacity, facilitating the progression of tubal intraepithelial carcinoma (TIC) (Figure 6). This second pathway leads to formation of Kurman's and Shin's "Type II" tumors.

Figure 6. Fallopian tube as a site of origin (KARST and DRAPKIN, 2010). The fallopian tube epithelium (FTE) is composed of a single layer of ciliated and secretory cells that are exposed to ovulation-associated inflammatory cytokines and reactive oxygen species (ROS). Repetitive genotoxic stress causes DNA damage and induces p53 mutation, leading to the clonal expansion of normal looking FTE cells of secretory phenotype. These damaged cells stain strongly for p53 ("p53 signature"). Further genetic "hits" enable cells to acquire a proliferative capacity, giving rise to tubal intraepithelial carcinoma (TIC). As a TIC progresses to invasive serous carcinoma, malignant cells are exfoliated from the fimbria and they may spread rapidly to the surface of the peritoneum and/or ovary. Exfoliation may also occur from TICs prior to fimbrial invasion.

2.7 Current treatment of EOC and resistance

The bilateral salpingo-oophorectomy (BSO) is generally recommended for women with high risk of developing ovarian cancer (with a known germline mutations of BRCA1 and BRCA2) at approximately 35 to 40 years of age as apart of the prophylactic treatment (NCCN, 2005). Although prophylactic BSO reduces the risk of ovarian cancer by more than 90%, high-risk patients with BRCA1 and BRCA2 mutations can still develop primary peritoneal cancer, which develops in 4% to 5% of women at 20 years after BSO (FINCH *et al.*, 2006).

The standard treatment for early-stage ovarian cancer patients is typically cytoreductive surgery. Stage I patients with favourable prognosis factors have more than 90% cure rate with surgery alone. Early-staged patients with unfavourable prognostic features (such as poorly differentiated tumors or evidence of spread) have 70 to 80% cure rate (OZOLS *et al.*, 2004).

After completion of surgery, patients with advanced disease require chemotherapy or radiotherapy. EOC is considered to be a chemosensitive neoplasm, with initial overall response rates to systemic therapy exceeding 80% when integrated with surgery. However, among women with advanced stage disease at diagnosis, long term survival remains poor due to eventual tumor recurrence and emergence of drug-resistant disease. Combination chemotherapy using paclitaxel with a platinum-based regimen is currently the standard first-line treatment for ovarian cancer (COLOMBO *et al.*, 2006). Whereas cisplatin-paclitaxel combination chemotherapy has shown significant efficacy over previous drug combinations, 20-30% of patients fail to respond to this combination and 90% of patients that initially responded to therapy will eventually develop chemotherapy-resistant disease (MCGUIRE *et al.*, 1996). Patients that do not respond to the first-line chemotherapy are given second-line and third-line regimens of chemotherapy in an attempt to prolong life and palliate symptoms. Second-line chemotherapeutic agents are rarely curative, with initial response ranging from 10-33% (JUDSON *et al.*, 1999). The discovery of novel and effective therapy against chemotherapy-resistant ovarian cancer remains a high priority.

2.7.1 Mechanisms of resistance to chemotherapy

A wide range of metabolic or structural properties within tumour cells may lead to drug resistance. The identified mechanisms include: decreased drug uptake, increased drug efflux, increased repair of DNA damage induced by chemotherapy and reduced ability to undergo apoptosis (GOLDENBERG *et al.,* 1998). In addition, because cancer cells are heterogeneous, more than one mechanism of drug resistance may be present in any particular case.

Initially sensitive tumor cells may become resistant to drugs during repetitive chemotherapy, which is so called *acquired (induced) resistance*, or they may be resistant without previous exposure to drugs- *intrinsic resistance.* Cells with acquired drug resistance can be produced experimentally by the successive exposure of parent cells to a particular drug *in vitro* (BOSCH and CROOP, 1996; Lane *et al.,* 2006). Several mechanisms of acquired drug resistance have been demonstrated, including reduced accumulation of drug, changes in the expression of enzymes involved in the glutathione detoxification pathway, alterations in DNA repair, cell cycle checkpoints and ratio of pro and anti-apoptotic Bcl-2 family members (Figure 7). In contrast to acquired drug resistance, few studies have examined the mechanisms responsible for intrinsic drug resistance. The reason why some tumor cells are inherently resistant without previous drug treatment is not known. In addition, importance of the *de-novo (environment-mediated)* drug resistance has been acknowledged. This type of resistance is mediated by tumor microenvironment which represents a rich source of both soluble factors and components of ECM, both of which can favour cell survival following drug exposure. Mechanisms associated with de-novo drug resistance may contribute to the failure to eliminate minimal residual disease and facilitate the emergence of acquired drug resistance (MEADS *et al.,* 2009; LANE *et al.,* 2010; KERBEL *et al.,* 1994).

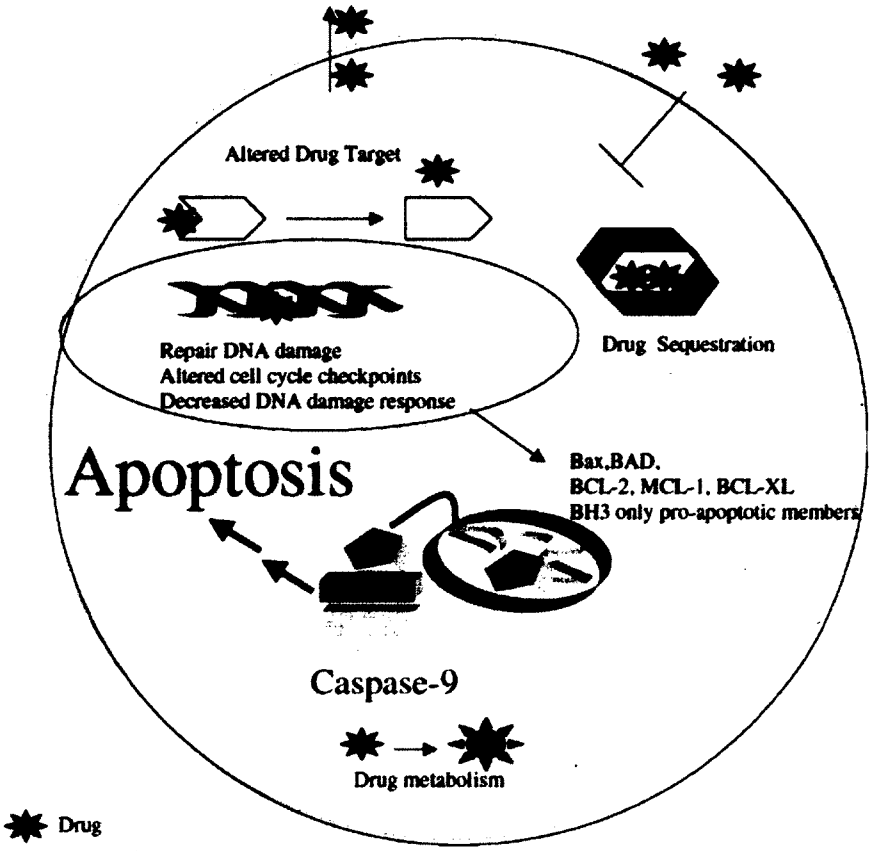

Figure 7. Acquired drug-resistant models possess multiple drug-resistance mechanisms (HAZLEHURST *et al.*, 2003). These mechanisms include: (a) decreased intracellular concentration of the drug characteristic of overexpression of drug transporters; (b) alterations in the drug target such as point mutations or overexpression of the target; (c) increased detoxification of the drug such as glutathione conjugation; (d) changes in the repair of DNA damage induced by the drug (e) alterations in the cell cycle checkpoint such as p27Kipl or p21; (e) changes in the ratio of pro and anti-apoptotic Bcl-2 family members.

The ability of most types of cancer to metastasize was also linked to their capacity to express resistance to anti-cancer drugs, including all major classes of drugs used in chemotherapy. Expression of certain dominantly acting oncogenes or altered expression of tumor suppressor genes can enhance not only tumor cell growth and malignant

aggressiveness, but also the relative expression of drug resistance (HENNEQUIN *et al.*, 2003). Some of these genetic alterations, mutations of suppressor gene p53 being a good example, often occur in more advanced stages of the disease (LASSAM *et al.*, 1993) and are relevant to acquisition of malignant properties and also drug resistance.

Interestingly, a number of studies demonstrated connection between increased activation of the pro-survival PI3K/Akt pathway in ovarian cancer cells and resistance to chemotherapy. *In vivo* and *in vitro* studies demonstrated that inhibition of PI3K enhanced paclitaxel-induced apoptosis in the human ovarian cancer cells (HU *et al.*, 2002). XIAP (X-linked inhibitor of apoptosis protein), Akt2 and p53 are important mediators of chemoresistance in EOC. Inhibition of XIAP and/or Akt expression/function is considered to be effective of overcoming chemoresistance in EOC cells (FRASER *et al.*, 2003). Targeting PI3K/Akt pathway has been shown to enhance sensitivity to docetaxel in breast and ovarian cancer (XING *et al.*, 2008). Recent study demonstrated that Akt confers resistance to cisplatin, by modulating cisplatin-induced, p53-dependent c-FLIP (FLICE-inhibitory protein) ubiquitination (ABEDINI *et al.*, 2009). Combined treatment of carboplatin and PI3K inhibitor LY294002 has been demonstrated to effectively decrease ovarian tumor progression (WESTFALL and SKINNER, 2005). However, another study indicates that efficacy of LY294002 may be greatly affected by the tumor p53 status (BAR *et al.*, 2005).

2.7.2 Targeted therapies for ovarian cancer

Despite evidence of considerable heterogeneity in their histological phenotypes and molecular profiling, most cases of ovarian cancer are treated in a similar fashion. It became apparent that the focus should be towards the development of new targeted therapies capable of exploiting molecular and genetic characteristics of individual tumor subtypes. At the moment, there is rapid development of novel compounds, including antiangiogenic reagents, humanized monoclonal antibodies, selective hormonal agents, and small molecules that target key components in signal transduction pathways associated with cell growth, tumor vascularity, and invasive potential.

A number of studies have focused on the epidermal growth factor receptor (EGFR), which is overexpressed in 30 to 70% of EOC (BASELGA, 2002). EGFR inhibitors such as cetuximab, ABX-EGF, erlotinib have been evaluated in clinical trials (FINKLER *et al.*, 2001). In spite of encouraging preclinical models, early results from large trials have not shown a benefit (OZOLS *et al.*, 2004).

Vascular endothelial growth factor (VEGF) has a key role in ascites formation. Antibody against VEGF has been shown to prevent and even reverse ascites formation in preclinical studies on mice (HU *et al.*, 2002). Bevacizumab (Avastin) is a recombinant humanized monoclonal antibody against VEGF, which has shown activity in ovarian cancer. Bevacizumab was given as a single agent to the women with recurrent ovarian or primary peritoneal cancer. 21% of women responded to therapy and 40% of patients had progression-free survival for 6 months and more (BURGER *et al.*, 2005). However, a serious adverse effect (bowel perforation) has been reported due to use of Bevacizumab (HEINZERLING and HUERTA, 2006).

Replacing defective genes that cause malignant behaviour of cancer cell is another approach. One such target is TP53. Promising preclinical data from *in vitro* systems led to phase I trials, but this trial was closed after first analysis due to the lack of any signs of efficacy (ZEIMET and MARTH, 2003). One of the possible problems is the presence of adenovirus-neutralizing antibodies in EOC ascites. Another study demonstrated that adenoviral transduction of primary ovarian cancer samples was abolished by autologous ascitic fluids (INGRAM *et al.*, 2010).

It is now recognized that in addition to genetic alterations, epigenetic mechanisms, such as DNA methylation, histone modifications and nucleosome remodeling, play an important role in the development and progression of ovarian cancer by modulating chromatin structure, and gene and miRNA expression. Furthermore, epigenetic alterations have been recognized as useful tools for the development of novel biomarkers for diagnosis, prognosis, therapeutic prediction and monitoring of diseases. Moreover, new epigenetic therapies, such as DNA methyltransferase inhibitors and histone deacetylase inhibitors,

have been found to be a potential therapeutic option for ovarian cancer, especially when used in combination with other agents (MARADEO and CAIRNS, 2011; MALDONADO and HOQUE, 2010).

3. TRAIL as a promising agent for cancer treatment

Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) is a member of the TNF superfamily. Because of its unique ability to trigger apoptosis (programmed cell death) in cancer cells and spare normal cells, TRAIL is a promising agent for cancer therapy. Deregulated apoptosis not only plays a key role in the pathogenesis and progression of cancer, but also leads to resistance to chemo and radiotherapy. Chemo and radiotherapy works effectively in killing some cancer cells through the intrinsic apoptotic pathway engaged by activation of p53 in response to DNA damage, but many tumors in which p53 is mutated or deleted are less sensitive to chemo or radiotherapy (LOWE *et al.*, 1994). In addition, the chemo or radiotherapy induces apoptosis in both cancer and normal cells and therefore possesses severe toxic side effects.

Members of TNF family including Fas ligand, TNF and TRAIL have been identified as important targets for cancer biological therapy (ASHKENAZI, 2002). Administration of Fas Ligand or TNF can induce apoptosis in different cancer cells but cause severe toxicity to liver, limiting their application in the clinic (OGASAWARA *et al.*, 1993; DUIKER *et al.*, 2006). TRAIL appears as a promising cancer therapeutic agent and induces apoptosis in wide variety of tumor cells without causing toxicity to normal cells (ASHKENAZI *et al.*, 1999). Importantly, TRAIL triggers apoptosis in cancer cells, regardless of p53 status, and therefore is an attractive agent especially for cancer cells in which p53 function has been inactivated, because it helps to overcome resistance to chemo and radiotherapy.

3.1 TRAIL and its receptors

Cells of immune system such as natural killers, T cells, macrophages and dendritic cells express TRAIL. TRAIL is inserted in the cell membrane with its C-terminal domain

exposed and has molecular weight of 33 kDa. It can be processed by cysteine proteases to produce soluble TRAIL of 20 kDa and both forms can trigger apoptosis by interacting with TRAIL receptors present in target cells. Five receptors that bind TRAIL have been identified so far (Figure 8). Only two of them, death receptor 4 (DR4) or TRAIL-R1 and DR5 or TRAIL-R2 are able to transmit an apoptotic signal (SHERIDAN *et al.*, 1997). Two additional so called decoy receptors DcR1 or TRAIL-R3 and DcR2 or TRAIL-R4 are incapable of transmitting an apoptotic signal (DEGLI-ESPOSTI *et al.*, 1997). DR4 and DR5 contain death domains (DD) in their intracellular portion. DcR1 lacks DD and instead possesses a glycosylphosphatidylinositol (GPI) anchor. DcR2 has a truncated DD. TRAIL also binds a soluble receptor called osteoprotegerin (OPG), which is involved in regulation of bone formation, but it has low affinity to TRAIL (TRUNEH *et al.*, 2000). The selective killing of tumor cells by TRAIL has been explained by higher level of DR4 and DR5 expression in tumor cells and relative high level of decoy receptors in normal cells (PAN *et al.*, 1997).

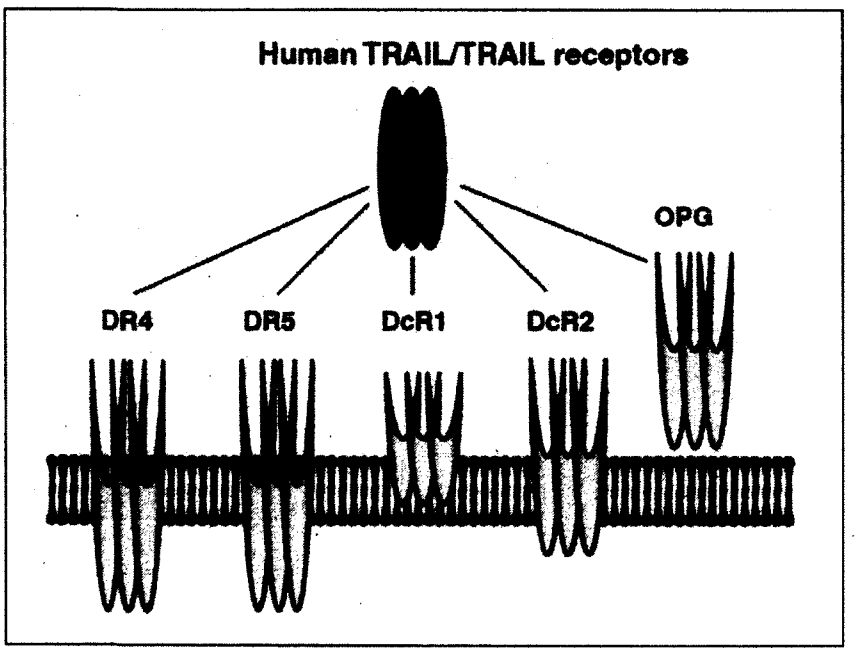

Figure 8. TRAIL and its receptors (WANG, 2008). There are 2 pro-apoptotic TRAIL receptors (DR4 and DR5) and three anti-apoptotic receptors (DcR1, DcR2 and OPG).

3.2 The physiological role of TRAIL and its receptors

TRAIL-TRAIL receptor pathway regulates different physiological processes such as haematopoiesis (SECCHIERO and ZAULI, 2008), T-cell activation and survival (JANSSEN *et al.*, 2005), and has been involved in many pathophysiological conditions including asthma, autoimmune diseases, diabetes, inflammation and excessive host immune responses in bacterial meningitis. TRAIL contributes to the host immunosurveillance against primary tumor development and metastasis by boosting the host responses to the tumor and somehow changing the tumor microenvironment for enhanced antigen presentation and tissue infiltration (JOHNSTONE *et al.*, 2008).

3.3 Apoptotic TRAIL signaling

There are two main apoptotic pathways to initiate the programmed cell death including *extrinsic pathway* and *intrinsic pathway*, and both are triggered depending on the different stimuli (Figure 9).

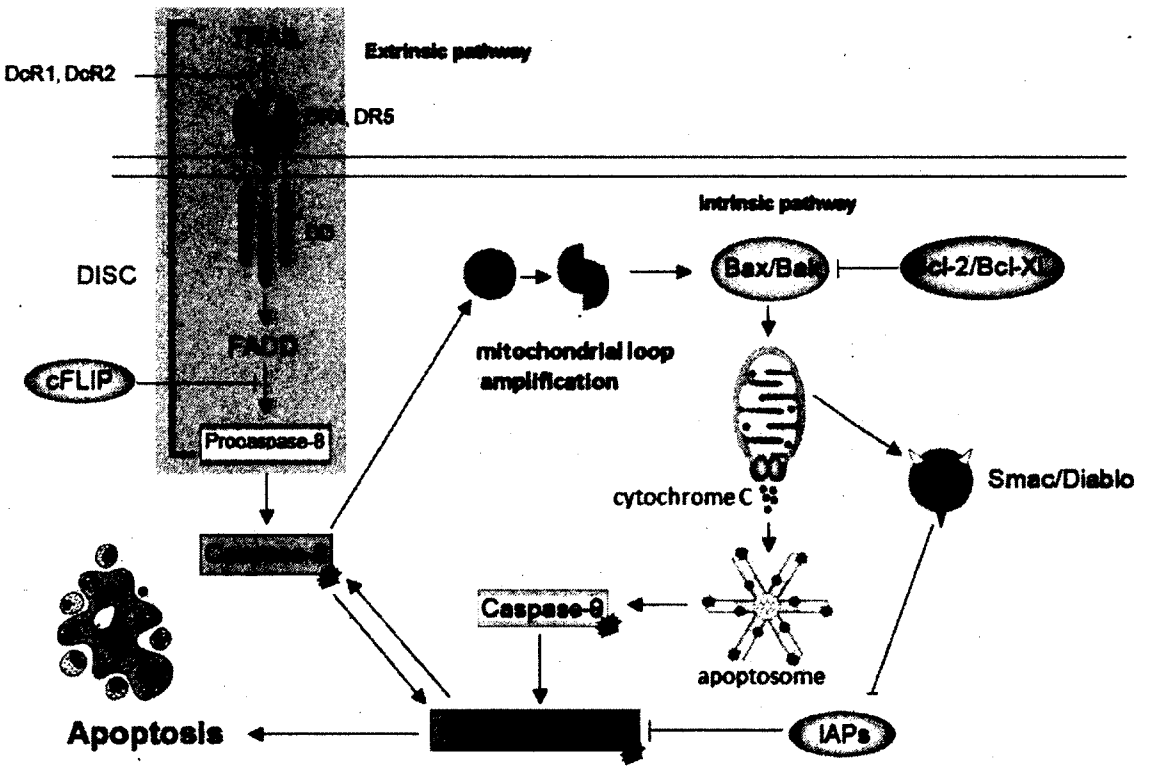

Figure 9. Apoptotic TRAIL signalling. Binding of TRAIL to death receptors (DR4, DR5) leads to a Bid-cleavage-dependent mitochondrial amplification step. Translocation of tBid to the mitochondria promotes the assembly of Bax-Bak oligomers and mitochondria outer membrane permeability changes. Cytochrome c is released into cytosol resulting in apoptosome assembly. Active caspase-9 then propagates a proteolytic cascade of effector caspases activation that leads to morphological hallmarks of apoptosis.

Extrinsic pathway triggers apoptosis when TRAIL binds to DR4 or DR5 death receptors. The trimerized receptors recruit the adaptor protein FADD (Fas-associated protein with death domain) that simultaneously binds the inactive pro-form of caspase-8 or caspase-10 via a shared death effector domain (DED) leading to formation of death inducing signaling complex (DISC). At the DISC pro-caspases are autoactivated by proteolysis. Activated caspase-8 or 10 then cleaves and directly activates the effector caspases (caspases-3,-6,-7) leading to the execution of apoptosis. FLIP (FLICE-inhibitory protein) has structural homology to pro-caspase-8, but lacks protease activity. Two splice variants of FLIP (long and short forms) were identified in human cells. This structure allows FLIP to bind to the DISC, thereby inhibiting the processing and activation of the initiator caspase-8 (Figure 9).

The *intrinsic pathway* is usually triggered in response to DNA damage, hypoxia or oncogene overexpression. As a sensor of cellular stress p53 is critical initiator for the intrinsic pathway. This pathway involves the activation of pro-apoptotic Bcl-2 family members Bax and Bak that form pores in the outer mitochondrial membrane causing the release of pro-apoptogenic factors such as cytochrome c and Smac/Diablo (second mitochondria-derived activator of caspase/direct IAP binding protein with low pI). The release of cytochrome c leads to the formation of the *apoptosome* formed by released cytochrome c, APAF1 (apoptotic protease activating factor-1) and the inactive initiator caspase pro-caspase-9. Within apoptosome caspase-9 gets activated and it cleaves downstream effector caspases, leading to the apoptosis (Figure 9).

The apoptotic self-destruction machinery is tightly controlled. Various proteins regulate the apoptotic process at different levels. IAPs (inhibitor of apoptosis proteins) can bind to and inhibit caspases. IAPs are inhibited by Smac/Diablo, which is released from mitochondria along with cytochrome c during apoptosis and it displaces IAPs from caspases (Figure 9). But the most important regulators of apoptosis at the mitochondrial level are apoptotic proteins that belong to the Bcl-2 family which control permeabilization of the outer mitochondrial membrane and release of cytochrome c and Smac/Diablo (BRUNELLE, 2009). Once activated, caspase-8 cleaves pro-apoptotic Bcl-2 family member protein Bid and active truncated Bid (tBid) interacts with pro-apoptotic Bax/Bak. This interaction leads

to the increased release of cytochrome c from mitochondria. Bid activation provides a connection between extrinsic and intrinsic pathways called the *mitochondrial loop amplification* (Figure 9).

In so called *type I cells*, activation of caspase-8 is sufficient to directly activate the downstream effector caspases leading to the apoptosis. The extrinsic pathway in type I cells is independent of mitochondria and could not be blocked by Bcl-2, whereas in *type II cells* such as ovarian cancer cells, the amount of caspase-8 or -10 is not enough to trigger the activation of effector caspases and apoptosis is initiated through the mitochondrial loop amplification (OZOREN and EL-DEIRY, 2002).

3.3.1 Members of the Bcl-2 family

It has been well established that Bcl-2 family proteins are involved in regulating apoptosis by controlling mitochondrial membrane permeability. Several studies have demonstrated that these proteins can interact with each other and these interactions can neutralize their pro- or anti-apoptotic functions. The balance between anti- and pro-apoptotic members of Bcl-2 family dictates the fate of cell survival or death. The Bcl-2 family can be divided into pro-apoptotic and anti-apoptotic proteins. These proteins contain one or more Bcl-2 homology domains (BH), which share sequence homology (BRUNELLE and LETAI, 2009; CORY *et al.*, 2003; DANIAL and KORSMEYER, 2004). Most anti-apoptotic proteins contain BH domains 1-4 like Bcl-2, Bcl-XL, Bcl-w, Boo/Diva, and Mcl-1 (Figure 10).

Pro-apoptotic proteins can be divided into 2 groups according to their function and the number of BH domains possessed. BH domains 1-3 containing proteins are known as multidomain pro-apoptotic or effector proteins such as Bax, Bak, and Bok (Figure 10).

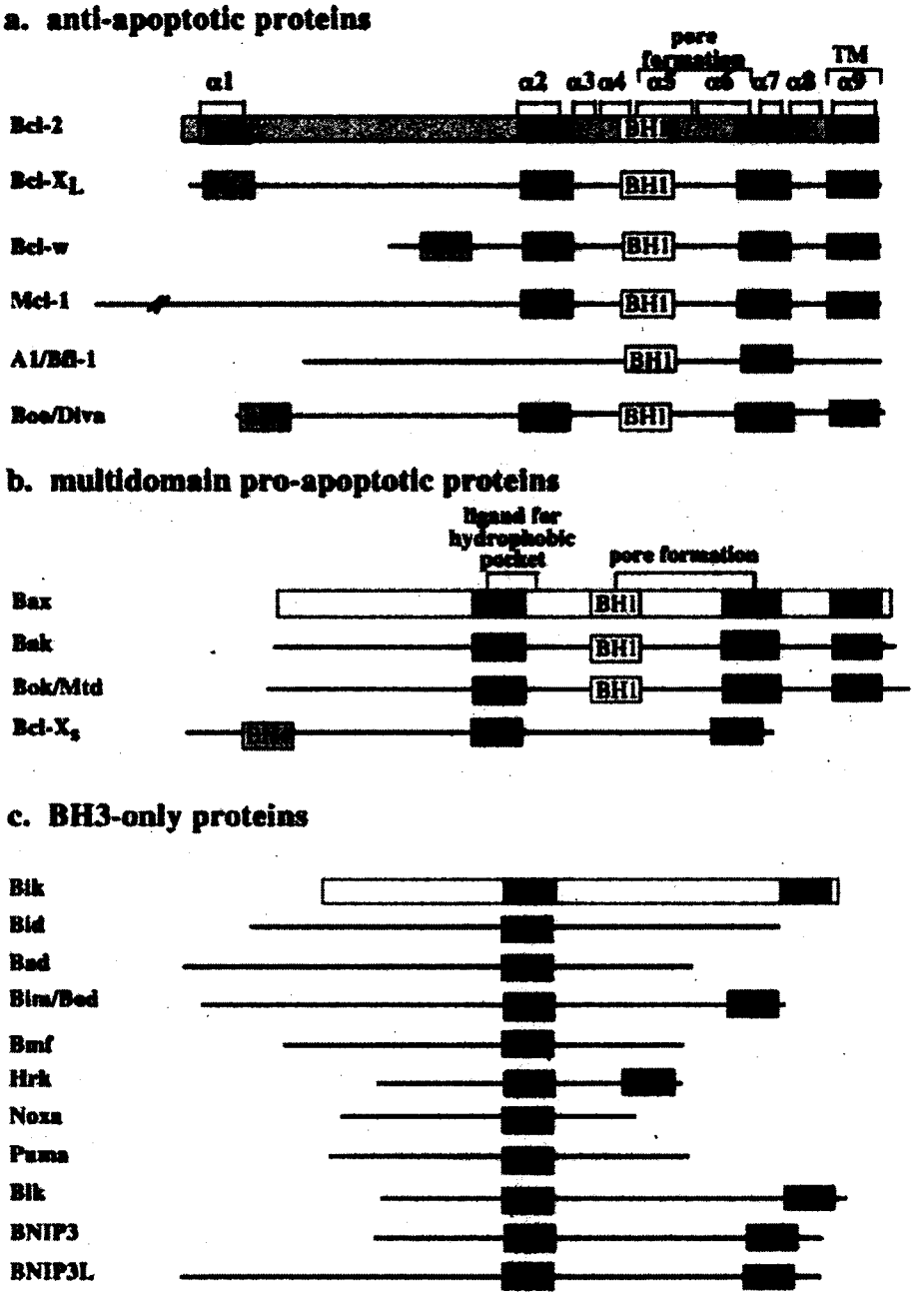

Figure 10. Members of Bcl-2 family (ER *et al.*, 2006). Representation of all known mammalian Bcl-2 family members. Bcl-2 homology regions 1-4 (BH1-4) are indicated. TM indicates the transmembrane region that mediates localization to intracellular membranes. Hα5-Hα6 overlapping region containing BH1 domain corresponds to the pore-forming region. The BH3 domain in the pro-apoptotic members is a ligand for the hydrophobic pocket formed by the BH1-BH3 domains of the anti-apoptotic members.

The remaining pro-apoptotic proteins contain only BH3 domain and called BH3-only proteins like Bik, Bid, Bad, Bim, Bmf, Noxa, Puma, Blk and others. Most of these proteins contain a C-terminal hydrophobic α-helix, which is a potential transmembrane domain involved in their localization to the membranes of organelles such as the mitochondria, the ER and the nucleus. These proteins can form homo-dimers and/or hetero-dimers (ER *et al.*, 2006; OLTVAI *et al.*, 1993; YANG *et al.*, 1993), essentially through the interaction of their BH3 domain (CHITTENDEN *et al.*, 1995).

The BH4 domain of anti-apoptotic proteins is implicated in the control of their anti-death functions (SATTLER *et al.*, 1997; HUANG *et al.*, 1998). The molecular surface of the multidomain anti-apoptotic proteins contains BH3 binding grooves, which can accommodate BH3 domains from the pro-apoptotic members, resulting in highly variable affinities for each specific pair of interactions (LEVINE *et al.*, 2008).

Multidomain pro-apoptotic proteins Bax and Bak can form homo-dimers and hetero-dimers with BH3-only proteins such as Bid which induces their pro-apoptotic function. Bax and Bak can also form hetero-dimeric interactions with anti-apoptotic proteins such as Bcl-2. The α-helical conformation of anti-apoptotic Bcl-2 inserts into the hydrophobic groove of pro-survival Bax or Bak, thereby neutralizing the activity of multidomain pro-apoptotic proteins Bax and Bak to promote apoptosis (VAN DELFT and HUANG, 2006; LIU *et al.*, 2003; SATTLER *et al.*, 1997; PETROS *et al.*, 2000).

Anti-apoptotic proteins can form homo-dimers and also hetero-dimers with pro-apoptotic BH3-only proteins such as Bid or Bad and this interaction results in neutralizing of pro-survival function of anti-apoptotic Bcl-2 proteins. Several quantitative studies have indicated that Bim, Puma, and tBid (the truncated form of Bid, activated by caspase-8) bind avidly to all pro-survival proteins. The others pro-apoptotic proteins associate only with certain anti-apoptotic Bcl-2 family members. For example, Noxa engaged only with Mcl-1 and A1 leads to neutralization of the anti-apoptotic function of Mcl-1 and A1. Bad engaged only Bcl-2, Bcl-XL, and Bcl-w also neutralizing their function (ADAMS and CORY, 2007;

CHEN *et al.*, 2005). These findings indicate that efficient apoptosis requires neutralization of multiple pro-survival proteins.

The principle site of action of apoptosis regulation by Bcl-2-like proteins is the mitochondrial membrane. Anti-apoptotic proteins (Bcl-2, Bcl-XL, Bcl-w, and Mcl-1) mainly reside in mitochondria, protecting these organelles against mitochondrial membrane permeabilization (MMP). In addition, they can reside in other cellular compartments and hinder the movements of pro-apoptotic proteins to mitochondria.

While pro-apoptotic Bak is normally associated with the outer mitochondrial membrane, Bax resides in the cytosol of healthy cells. Bax and Bak are the two principal proteins required for MMP. MMP result from conformational change of Bax and Bak and their full insertion into mitochondrial membranes as homo-oligomerized multimers. It leads to the formation of giant protein-permeable pores in the membranes leading to the release of the contents of the mitochondrial intermembrane space, including cytochrome c and Smac/Diablo into the cytosol that drives caspase activation and apoptosis (PALGLIARI *et al.*, 2005; KROEMER *et al.*, 2007).

Given the lethal consequences of Bax and Bak activation, knowing how their activation is controlled is the key to understanding how a cell makes the decision to undergo apoptosis. Two models of Bax and Bak activation exist: the indirect and direct models (Figure 11).

In *direct activation model*, a subgroup of BH3-only proteins, termed *activators*, are proposed to bind directly to Bax and Bak to promote their activation (KUWANA *et al.*, 2005). The remaining BH3-only proteins, termed as *sensitizers*, function by binding to the pro-survival proteins and freeing any bound Bim or tBid to directly activate Bax and Bak (ADAMS *et al.*, 2007; CHEN *et al.*, 2005).

The indirect activation model proposes that all BH3-only proteins function solely by binding to their pro-survival relatives, thereby preventing those guardians of cell survival from inhibiting Bax and Bak (WILLIS *et al.*, 2005; UREN *et al.*, 2005). Several recent

findings strongly challenge the indirect activation model (WEI *et al.*, 2000; CARTRON *et al.*, 2004; REN *et al.*, 2010).

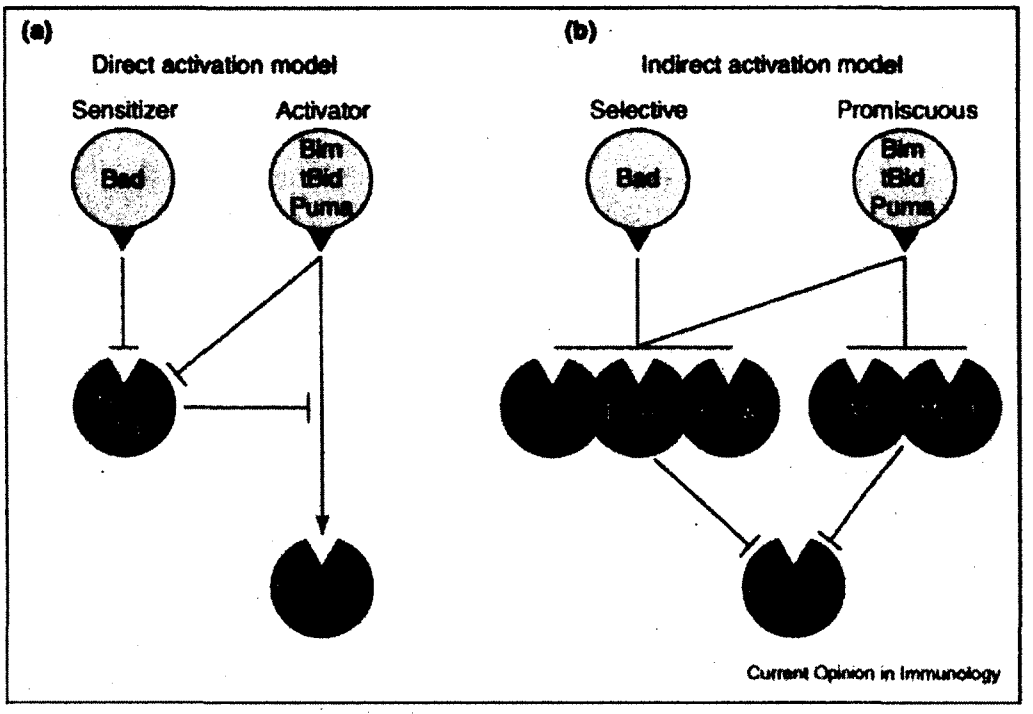

Figure 11. Models for Bax/Bak activation by BH3-only proteins (VAN DELFT and HUANG, 2006). **(A) Direct activation model.** BH3-only proteins can be divided into two groups: "sensitizers" or "derepressors" (e.g. Bad) that bind only to pro-survival proteins and "activators" (e.g. Bim) that can also directly engage Bax and Bak. "Sensitizers/derepressors" induce apoptosis by displacing "activators" from pro-survival proteins, and "activators" then proceed to trigger Bax/Bak activation. **(B) Pro-survival neutralization model.** Pro-survival proteins inhibit Bax and Bak, perhaps through direct interaction. BH3-only proteins induce apoptosis by neutralizing pro-survival molecules and Bax/Bak activation occurs spontaneously in the absence of pro-survival activity.

3.4 Additional signal transduction pathways activated by TRAIL

A number of studies show that TRAIL is also able to trigger non-apoptotic pathways (LIN *et al.*, 2000). Engagement of TRAIL receptors can lead to formation of a secondary signaling complex, probably after DISC assembly. This complex is formed by a part or all following proteins: FADD, active caspase-8, receptor interacting protein 1 (RIP1), TNF receptor associated protein (TRAF-2) (Figure 12).

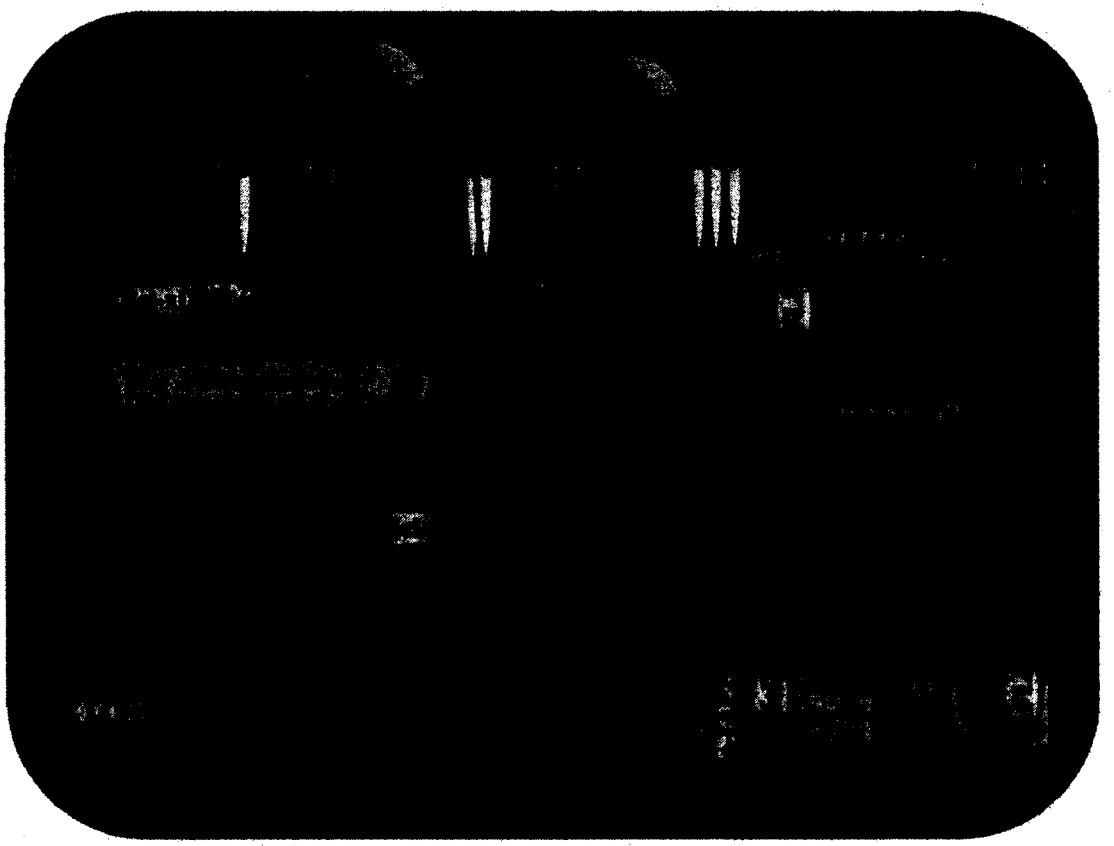

Figure 12. Alternative TRAIL signaling (WANG, 2008). The secondary signaling complex leads to the activation of NFkB and JNK and increased expression of anti-apoptotic or proliferation stimulatory proteins. The balance between pro- and anti-apoptotic signals varies between tumor cell types.

RIP1 links TRAIL receptor to stimulation of nuclear factor-kB (NFkB) activation, which in turn transactivates anti-apoptotic proteins Mcl-1, IAPs, Bcl-XL and c-FLIP leading to the inhibition of apoptosis (RICCI *et al.,* 2007). Mcl-1 can interact with tBid thereby preventing tBid-Bax/Bak interactions and blocks apoptosis (CLOHESSY, 2006). Recently it was demonstrated that Myc oncogene decreases the expression of Mcl-1 and IAPs by blocking TRAIL mediated NFkB activation (WANG, 2008; RICCI *et al.,* 2007). Myc also directly transactivates DR5 or suppresses the transcription of c-FLIP, therefore enhancing TRAIL-induced apoptosis (Figure 12).

TRAF2 leads to the JNK activation which promotes activation of pro-apoptotic protein Bim and results in apoptosis through the intrinsic mitochondrial pathway (CORAZZA *et al.,* 2006).

TRAIL can also activate p38 mitogen-activated protein kinase (MAPK) pathway (MASTERS *et al.,* 1996) and anti-apoptotic PI3K/Akt pathway (SECCHIERO *et al.,* 2003). Conflicting reports suggest that ERK, JNK or p38 signaling can either suppress or enhance the apoptotic efficacy of TRAIL (SECCHIERO *et al.,* 2003; Lee *et al.,* 2006; MUCHA *et al.,* 2009; WANG *et al.,* 2009; SHENOY *et al.,* 2009).

There is evidence that signal transduction mediated by TRAIL death receptors can be regulated by the localization of these proteins to cholesterol- and sphingolipid-rich *lipid rafts* within the plasma membrane (MUPPIDI and SIEGEL, 2004). Recently it was shown that ligation of DR4 and DR5 localized to lipid rafts induced a pro-apoptotic signal mediated by caspase-8 activation following DISC formation, but TRAIL receptors not associated with lipid rafts mediate the activation of NFkB, ERK1 and ERK2 (SONG *et al.,* 2007).

3.5 TRAIL receptor targeting agents

Recombinant soluble TRAIL and agonist antibodies targeting TRAIL receptors have been used to induce TRAIL-mediated apoptosis in tumor cells, with each approach having their own advantages and drawbacks.

In preclinical models, recombinant soluble TRAIL has demonstrated impressive anticancer activity. Importantly, no systemic toxicity was observed (WALCZAK *et al.,* 1999). The first form of recombinant TRAIL that has been taken into the clinic is AMG-951. Due to significant tumor penetration it possesses high anti-tumor activity *in vivo* (KELLEY and ASHKENAZI, 2004). Recombinant TRAIL may bind to all five TRAIL receptors, which means that it can activate pro-survival pathways in tumor cells which can create resistance to TRAIL-induced apoptosis. To avoid this unwanted effect, agonistic monoclonal antibodies (mAbs) that only target DR4 and DR5 were created. Some of them are Mapatumumab (anti-DR4), Lexatumumab (anti-DR5), and Apomab (anti-DR5). An advantage of mAbs compared to soluble TRAIL is that they have high affinity to their targets, thus limiting non-specific binding to decoy receptors or OPG. In addition, mAbs have a much longer half-life (around 15 days) than recombinant TRAIL (30 min) making them easier to dose and administer (DUIKER *et al.,* 2006). Moreover, mAbs can also recruit immune cells to the tumor site thus providing an additional means to antitumor activity (TAKEDA *et al.,* 2004). Interestingly, it has been demonstrated that DR5 selective variants of TRAIL were more potent than wild type TRAIL to trigger apoptosis in ovarian cancer cells (VAN DER SLOOT *et al.,* 2006).

Finally, gene therapeutic approaches with TRAIL-expressing adenoviral vectors are also being explored. Efficient tumor cell killing by adenoviral-expressed TRAIL has been demonstrated in several tumor cell lines and mice (ABOU EL HASSAN *et al.,* 2004; ZHANG *et al.,* 2005). However, this type of treatment had limitations, such as problems related to viral delivery and poor cell infection efficiencies.

3.6 TRAIL resistance

Despite promising results, studies have shown that number of cancer cells are resistant to TRAIL. TRAIL resistance has been reported in approximately 50 % of tested tumor cell lines reducing the expectations of monotherapy in the clinic (LEBLANC and ASHKENAZI, 2003).

Various mechanisms are used by tumors to escape apoptosis induction, but deregulation of a unique pathway for resistance to TRAIL has not been identified so far. Susceptibility to TRAIL-induced apoptosis can be regulated at several levels in the apoptotic signaling pathway. Some of the commonly known resistance mechanisms have been described (Figure 13).

It has been reported that lack of expression of DR4 due to epigenic silencing correlated with resistance to TRAIL-induced apoptosis in ovarian cancer cells (HORAK *et al.*, 2005). Aside from the surface expression, post-translational modifications of the death receptors can reduce their ability to transduce TRAIL signal. O-glycosylation promotes ligand-stimulated clustering of DR4 and DR5, which mediates recruitment of caspase-8 and apoptosis activation (WAGNER *et al.*, 2007). S-palmitoylation of DR4 has also been identified as a pre-requisite for its localization to lipid rafts as well as for its ability to provide TRAIL-induced signalization (ROSSIN *et al.*, 2009).

Sensitivity to TRAIL can depend on ratio of TRAIL-decoy to death receptors. Decoy receptors can inhibit TRAIL-mediated apoptosis by competing with DR4 and DR5 for binding (MÉRINO *et al.*, 2006). Also, mutated DR5 can similarly compete for ligand binding by acquiring a decoy receptor function (BIN *et al.*, 2007).

Resistance to TRAIL has also been correlated with high levels of c-FLIP that inhibits activation of caspase-8 at the DISC in several cancer types including breast, lung, colon

and ovarian cancer (GUSEVA *et al.*, 2008; WANG *et al.*, 2008; DOLCET *et al.*, 2005; TOMEK *et al.*, 2004; LANE *et al.*, 2007).

The ratio of pro-versus-anti-apoptotic Bcl-2 members is crucial for induction of intrinsic pathway of apoptosis. Resistance to TRAIL has been associated with increased levels of anti-apoptotic Bcl-XL (SONG *et al.*, 2007), Mcl-1 (WANG *et al.*, 2008; TANIAI *et al.*, 2004) and Bcl-2 (ZHANG and FANG, 2005) and decreased expression or mutations of pro-apoptotic members such as Bax, Bak and Bid (RAVI and BEDI, 2002; JONG *et al.*, 2004; VAN GEELEN, 2004; KANDASAMY *et al.*, 2003; CHEN *et al.*, 2001).

High IAPs expression has been indicated as another mechanism of TRAIL resistance in different types of cancer, including ovarian cancer (CHAWLA-SARKAR *et al.*, 2004; LEE *et al.*, 2006; LIPPA *et al.*, 2007).

The pro-survival PI3K/Akt pathway has been shown to have negative impact on TRAIL-induced apoptosis through its ability to increase the expression of anti-apoptotic proteins such as c-FLIP (PANNER *et al.*, 2005), XIAP (SHRADER *et al.*, 2007) and Bcl-2 (CHEN *et al.*, 2001). A number of studies have demonstrated that tumor cells with activating somatic mutations in PI3K are relatively resistant to apoptosis triggered by TRAIL or chemotherapy (ZHANG *et al.*, 2009; CAMPBELL *et al.*, 2004; YUAN *et al.*, 2000). TRAIL-resistant ovarian carcinomas have been shown to have increased activation of PI3K/Akt pathway (ALTOMARE *et al.*, 2004).

Figure 13. Mechanisms of resistance to TRAIL (MELLIER *et al.*, 2010). Resistance arises from deficiencies at different levels of the TRAIL signaling pathway: at the receptors level (expression, localization, mutation, competition, and endocytosis/transport), at the DISC level (caspase-8, c-FLIP) or at the level of the mitochondrial apoptotic pathway (regulation of Bcl-2 family members, Smac/DIABLO, IAPs).

3.7 Resistance to TRAIL-induced apoptosis among ovarian cancer cells

Previous studies have demonstrated that ovarian cancer cell lines and primary ovarian tumor cells isolated from malignant EOC ascites displayed variable sensitivity to TRAIL. TRAIL-mediated apoptosis was deficient in nearly 50% of ovarian cancer cell lines and primary tumors (LANE *et al.*, 2004; CUELLO *et al.*, 2001).

Resistance to TRAIL therapy can be subdivided into a three categories: *acquired*, *de-novo* and *intrinsic* resistance (Figure 14). We demonstrated that *acquired* (induced) resistance to TRAIL-induced apoptosis in human ovarian cancer cells is conferred by increased turnover of mature caspase-3 (LANE *et al.*, 2006).

We have also investigated the *de-novo* resistance by treating EOC cells with ascites and demonstrated that EOC ascites, which represent a unique form of tumor microenvironment (Figure 3), protect TRAIL-sensitive CaOV3 cells from TRAIL-induced apoptosis by activating the PI3K/Akt pathway (LANE *et al.*, 2007). In addition, we established that the PI3K/Akt pathway is activated by ascites through the αvβ5 integrin-mediated focal adhesion kinase activation (LANE *et al.*, 2010).

Nearly 50 % of EOC cell lines are intrinsically resistant to TRAIL-induced apoptosis meaning that these cells do not respond to the therapy the first time introduced to TRAIL. The mechanisms of intrinsic resistance to TRAIL in EOC cells are largely unknown. Our laboratory has previously shown that TRAIL induces apoptosis in only limited number of ovarian cancer cell lines (only two out of seven cell lines tested). Primary ovarian carcinoma cells display variable sensitivity to TRAIL. The lack of response to TRAIL was associated with limited activation of the apoptotic cascade in cell lines resistant to TRAIL (LANE *et al.*, 2004).

Although there are a number of mechanisms that can be altered in EOC cells that lead to intrinsic resistance to TRAIL, the PI3K/Akt pro-survival pathway is of particular interest because it has been implicated as a major determinant of oncogenic transformation in

ovarian cancer. PI3K is up-regulated in 30 to 45% of ovarian cancer cell lines (SHAYESTEH *et al.*, 1999; CAMPBELL *et al.*, 2004), whereas about 70% of primary tumors show elevated Akt activity (ALTOMARE *et al.*, 2004).

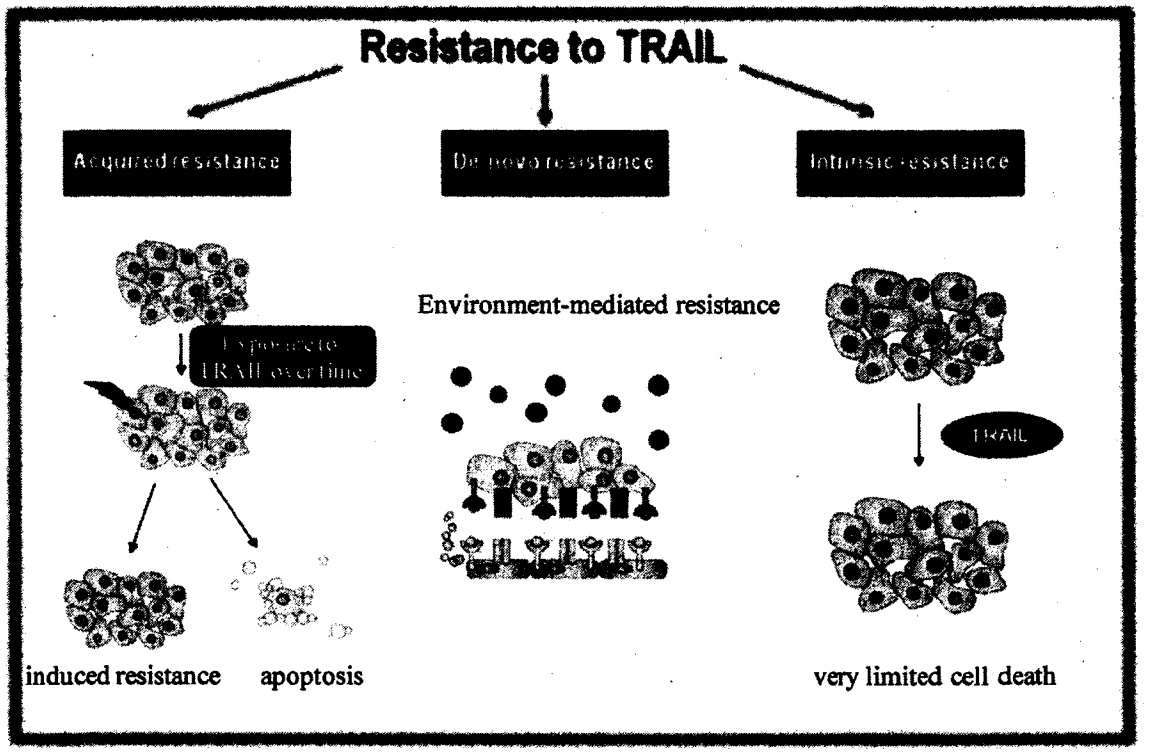

Figure 14. Resistance to TRAIL. a) *Acquired* (induced) resistance develops over the time from exposure to sub-lethal TRAIL concentration. This type of resistance is associated with occurrence of sequential genetic alterations in the cells that eventually result in therapy-resistant phenotype (MEADS *et al.*, 2009). b) *De-novo* is environment-mediated resistance, in which tumor cells are transiently protected from TRAIL-induced apoptosis. This form of resistance is rapidly induced by signaling events that are initiated by factors present in the tumor microenvironment (KASSIS *et al.*, 2004). c) *Intrinsic* resistance: some tumor cells are inherently resistant even to high concentrations of TRAIL without previous exposure to the agent.

4. PI3K/Akt pathway

4.1 PI3K/Akt pathway components and activation mechanisms

Phospatidylinositol-3 kinases, PI3Ks belong to a lipid kinase family that are activated in response to binding of growth factors and cytokines to receptors with protein tyrosine kinase activity (RPTK) or receptors coupled with G proteins. Once activated, PI3K phosphorylate the membrane lipid PIP2 (phospatidylinositol (4, 5)-biphosphate) into PIP3 (phospatidylinositol (3, 4, 5)-trisphosphate) (Figure 15). PIP3 is an important messenger that induces the activation of downstream target proteins through binding of their pleckstrin-homology (PH) domain that is found in many proteins, including the protein serine/threonin kinase 3-phosphoinositide-dependent kinase-1 (PDK-1) and the protein kinase B (PKB) also known as Akt. Akt interacts with PIP3 at the inner membrane where PDK-1 is located. PDK-1 activates Akt by phosphorylation (Figure 15). Activated Akt is translocated to various subcellular compartments, including the Golgi, endoplasmic reticulum, mitochondria and nucleus where it phosphorylates substrates or interacts with other molecules.

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is the lipid phosphatase that reduces PIP_3 levels in cell. PTEN specifically catalyses the dephosporylation of the 3` phosphate of the inositol ring in PIP_3, resulting in the biphosphate product PIP_2 (PtdIns(4,5)P2). By this action, PTEN negatively regulated PI3K/Akt pathway (CANTLEY and NEEL, 1999). PTEN has been shown to be mutated in 5 to 8% of ovarian carcinomas (KOLASA *et al.*, 2006; MUTTER, 2001), but in endometrioid carcinomas PTEN mutations were found in 20-26%. Interestingly, low grade endometriod carcinomas show higher frequency of PTEN mutations (80%) suggesting that PTEN mutations may play a role in a development of low-grade endometrioid tumors (KOLASA *et al.*, 2006). Despite the fact that PTEN mutations were found in less than 10% of ovarian carcinomas, PTEN protein inactivation has been found in 27% of ovarian carcinomas (KUROSE *et al.*, 2001) suggesting that transcriptional silencing by epigenetic mechanisms may be yet an additional means of modifying PTEN activity.

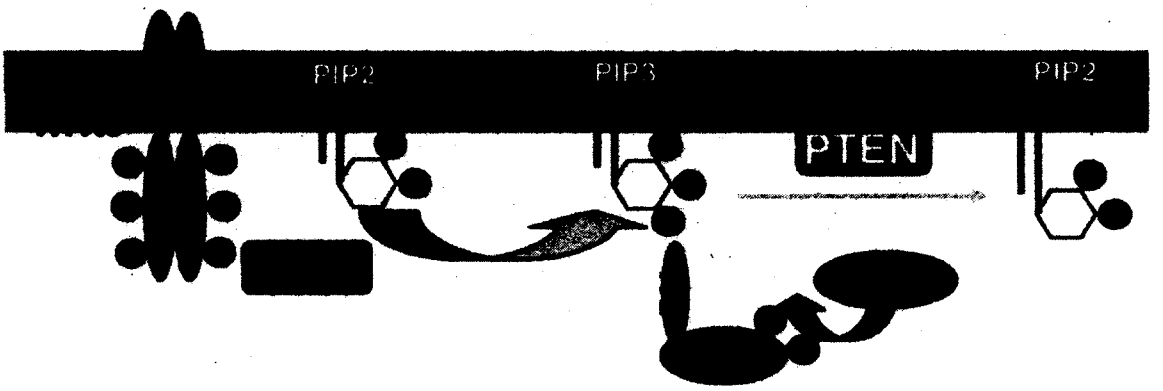

Figure 15. Mechanisms of Akt activation by PI3K. PI3K is induced as a result of receptor binding and activation of receptor tyrosine kinase (RTK). PI3K phosphorylates the membraine lipid PIP2 to PIP3. PTEN, the lipid phosphatase removes the phosphate from PIP3 and by this action blocks the receptor binding signal from propagating downstream. These downstream signals include a number of kinases that contain the pleckstrin homology (PH) domain such as PDK1 and Akt.

4.2 Akt/PKB

Akt was originally identified as the human homologue of the viral oncogene *v-akt* from transforming retrovirus AKT 8. Akt (PKB) is now classified as a family of kinases that has significant homology to protein kinase A (PKA) and protein kinase C (PKC).

To date, three members of this Akt family have been isolated, named Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ) (Figure 16). Although they are the product of different genes, they are closely related to each other, with up to 80% of amino acid homology (FRESNO VARA *et al.*, 2004).

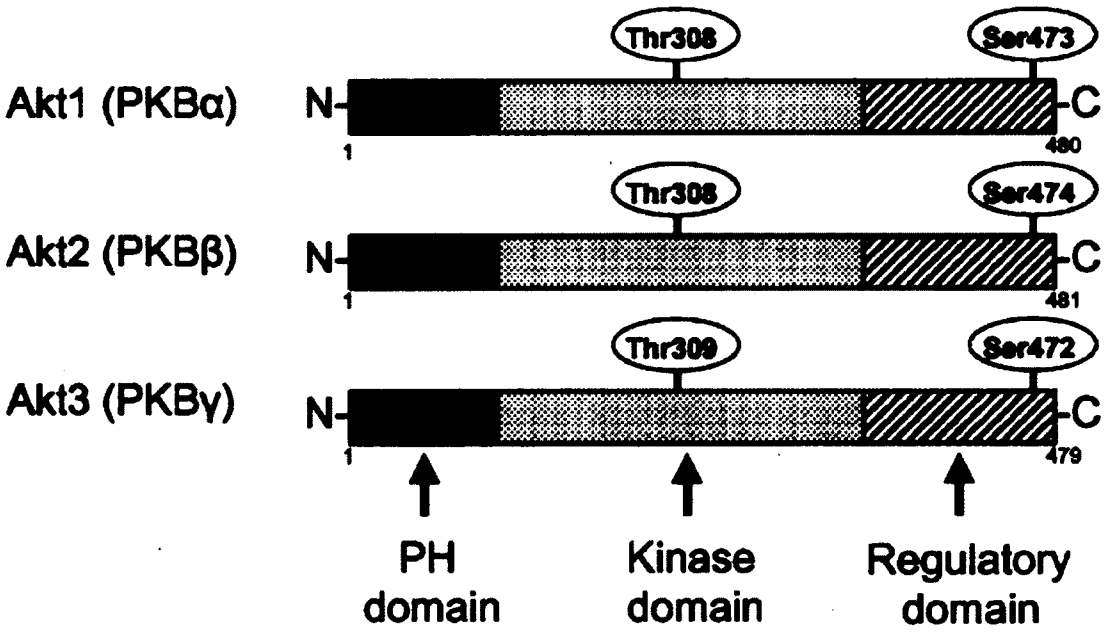

Figure 16. Three human Akt/PKB isoforms (OSAKI *et al.*, 2004). Each Akt isoform consists of three functional domains: an N-terminal PH domain for binding inositol phospholipids, a central kinase domain including Thr308 (Thr309 in Akt3), and a C-terminal regulatory domain including Ser473 (Ser474 in Akt2, Ser472 in Akt3). The number of amino acid residues is shown under each box.

All three mammalian Akt genes are widely expressed in various tissues (OSAKI *et al.*, 2004). Akt1 activity frequently elevated in breast and prostate cancers (SUN *et al.*, 2001). 36% of ovarian tumors exhibit elevated Akt2 activity (YUAN *et al.*, 2000; BELLACOSA *et al.*, 1995). Akt3 has been shown to be upregulated in estrogen receptor-deficient breast cancer cells and melanomas (STAHL *et al.*, 2004). Recent study demonstrated that Akt3 expression is detectable in 93% of primary ovarian tumors and specifically elevated in 20%. In this study different ovarian cancer cell lines that have been evaluated showed variable levels of expression of all 3 Akt isoforms (CRISTIANO *et al.*, 2006).

4.3 Cellular processes regulated by Akt

Akt plays an essential role in cell survival, growth, migration, proliferation, polarity, and metabolism (lipid and glucose), cell cycle progression, angiogenesis, and cell–renewal of stem cells (LIAO and HUNG, 2010).

4.3.1 Direct modulation of apoptosis by Akt

Akt inactivates by phosphorylation some pro-apoptotic factors: Bad, Bax, Bim and caspase-9 (Figure 17).

The pro-apoptotic *Bad* protein binds to anti-apoptotic Bcl-2 and Bcl-XL and blocks their anti-apoptotic functions. Activated Akt directly phosphorylates Bad on serine 136 (DATTA *et al.,* 1997). This phosphorylation of Bad by Akt results in the dissociation from Bcl-2 and Bcl-XL and binding of Bad to adapter protein 14-3-3 which results in Bad degradation (Figure 17).

When apoptotic signals are present, pro-apoptotic protein *Bax* is going through conformational changes which allow Bax to translocate to mitochondrial membrane and oligomerize to form large pores which allows release of cytochrome c and other pro-apoptotic factors from mitochondria (YAMAGUCHI and WANG, 2001). Phosphorylation of Bax by Akt on serine 184 results in inhibition of conformational change and inability of Bax to translocate to mitochondria. It was shown that phosphorylated Bax heteromerizes with Bcl-2 family members of anti-apoptotic function, such as Bcl-XL (GARDAI *et al.,* 2004).

Caspase-9 acts as an initiator of the apoptotic process as part of the apoptosome. Akt can also directly phosphorylate the human caspase-9 on serine 196, resulting in its inactivation (CARDONE *et al.,* 1998).

The pro-apoptotic activity of *Bim* can be regulated by Akt. A recent study has demonstrated that Akt can inhibit Bim functions by phosphorylating Bim on serine 87 (QI *et al.*, 2006).

Activation of stress-activated protein kinase (SAPK) pathway triggers mitochondria-dependent apoptosis in response to many types of stress. SAPK composed of two groups of kinases: JNK and p38 MAPK. Akt phosphorylates three kinases upstream of SAPK: ASK1 (apoptosis signal-regulating kinase 1) on serine 83 (KIM *et al.*, 2001), MLK3 (mixed lineage kinase 3) on serine 674 (BARTHWAL *et al.*, 2003) and MKK4 (mitogen-activated protein kinase kinase 4) on serine 78 (PARK *et al.*, 2002). Phosphorylation results in inactivation of these three proteins.

The *glycogen synthase kinase-3* (GSK-3) can phosphorylate the anti-apoptotic protein Mcl-1 which leads to its ubiquitination and facilitates cytochrome c release and apoptosis (MAURER *et al.*, 2006). GSK-3 is inactivated by Akt phosphorylation on serine 21 and 9 (CROSS *et al.*, 1995). Since Akt negatively regulates GSK-3 activation, it contributes to Mcl-1 stability and survival (Figure17).

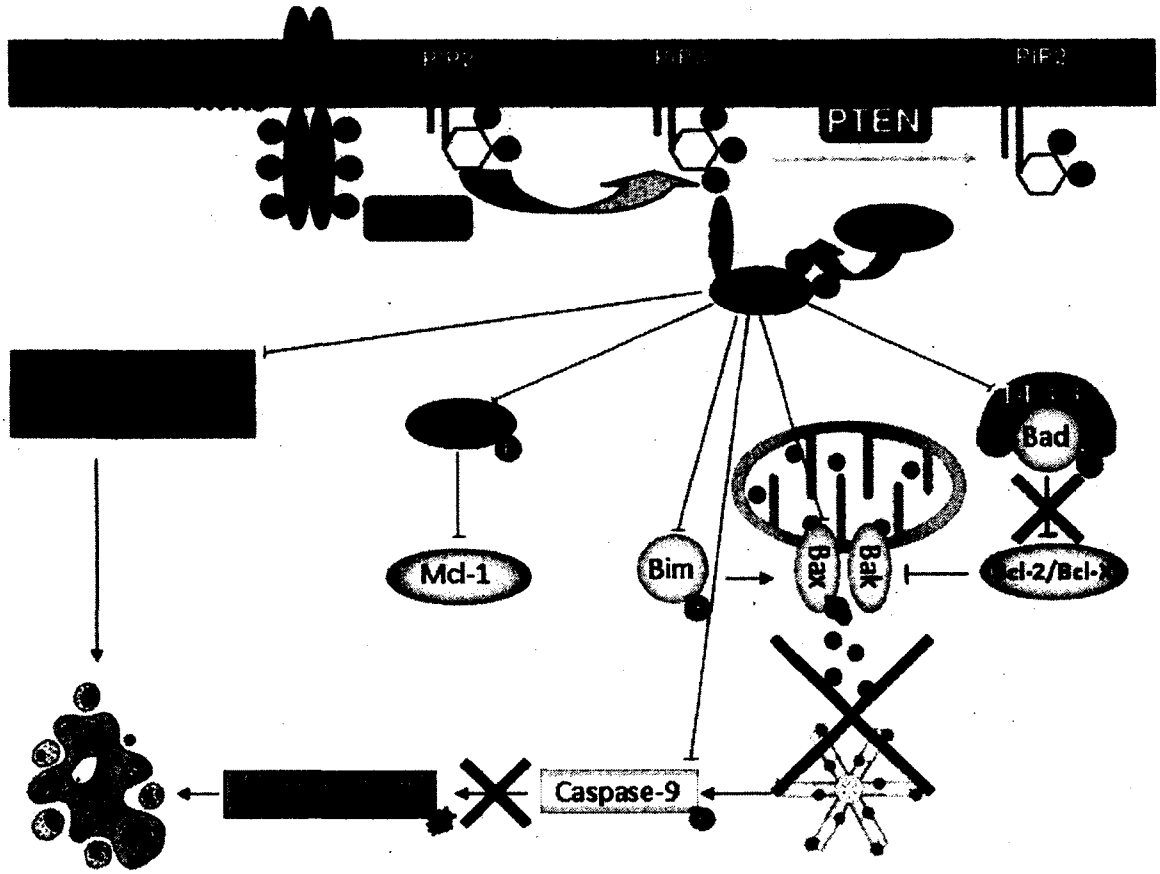

Figure 17. Direct modulation of apoptosis by Akt. The Bcl-2 family of proteins may represent a critical checkpoint in the mitochondrial intrinsic pathway of apoptosis and its members control cytochrome c release to the cytosol. By phosphorylating the pro-apoptotic molecule Bad, Akt induces its association with 14-3-3 proteins in the cytosol and thus contributes to the inhibition of Bad cell death functions. Akt also phosphorylates another pro-apoptotic member of the Bcl-2 family, Bax. Phosphorylated Bax promotes heterodimerization with anti-apoptotic Mcl-1 and Bcl-XL, leading to abrogation of Bax activity. Moreover, Akt has been found to phosphorylate and negatively regulate the Mcl-1 inhibitor, GSK-3. At the post-mitochondrial level, Akt may phosphorylate and inactivate caspase-9, resulting in the abolition of the caspase cascade. Akt also can abrogate activation of stress-activated protein kinase (SAPK) pathway.

4.3.2 Indirect modulation of apoptosis by Akt

The mediation of apoptosis execution by Akt also involves changes in the transcription program. Akt regulates transcription of factors responsible for the expression of pro- or anti-apoptotic molecules (Figure 18).

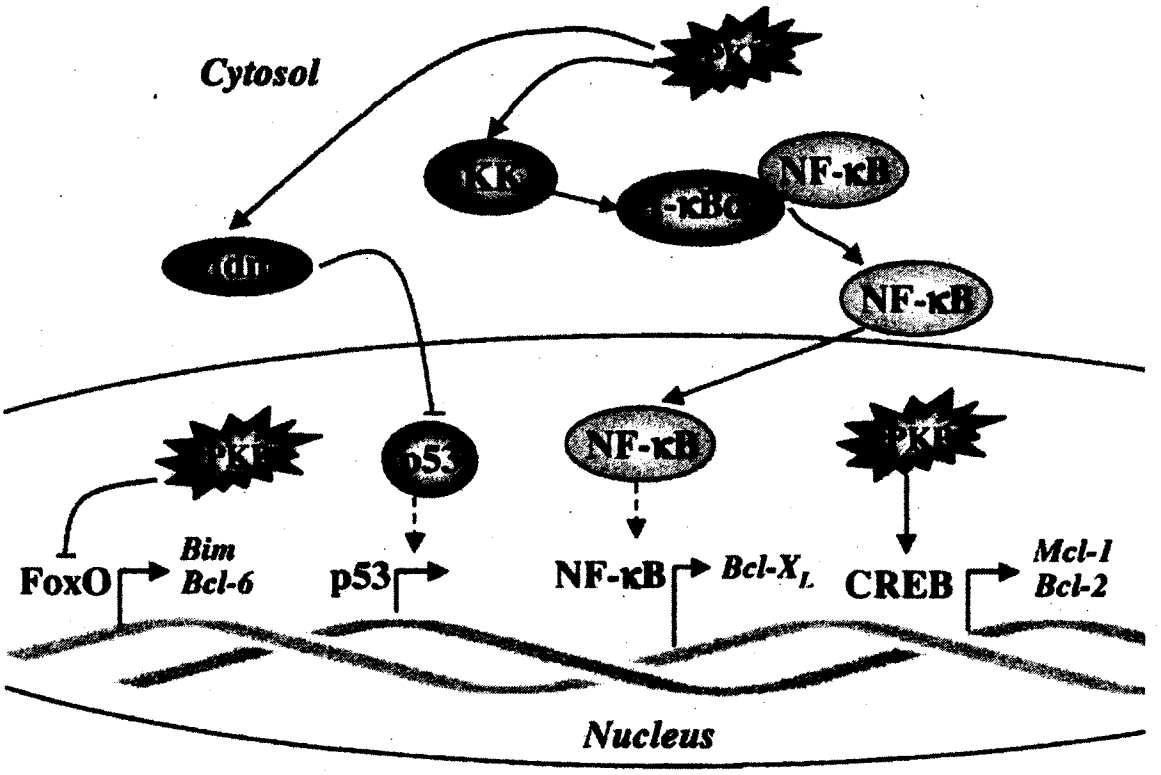

Figure 18. Indirect modulation of apoptosis by Akt (PARCELLIER *et al.*, 2008). PKB/Akt phosphorylates and inhibits FoxO and also activates CREB after its phosphorylation, resulting in the downregulation of pro-apoptotic and upregulation of anti-apoptotic Bcl-2 family members. Moreover, by phosphorylating and activating IKK, PKB/Akt induces I-κBα phosphorylation. Thus, NF-κB is activated and induces expression of Bcl-2 anti-apoptotic proteins. On the other hand, phosphorylation by PKB/Akt stabilizes Mdm2 and increases Mdm2 ubiquitin ligase activity, leading to a decrease in p53 transcriptional activity.

Forkhead family members: It has been shown that Akt is able to directly phosphorylate Forkhead protein (FoxO) and in this way it is responsible for their nuclear exclusion and inactivation (KOPS *et al.,* 1999). FoxO target genes are important for inhibition of cell survival and the promotion of apoptosis. For example, FoxO proteins regulate expression of pro-apoptotic Bcl-2 family members such as Bim or Bcl-6 (BURGERING *et al.,* 2003).

Murine double minute 2 (Mdm2): Mdm2 is an E3 ubiquitin ligase that negatively controls intracellular levels of the tumour suppressor p53. p53 is a major regulator of cell death and induces apoptosis by upregulating pro-apoptotic proteins PUMA and NOXA (NAKANO and VOUSDEN., 2001; SHIBUE *et al.,* 2003). It has been reported that Akt binds to and phosphorylates Mdm2, leading to its stabilization and allowing its nuclear import and increasing its ubiquitin ligase activity (GOTTLIEB *et al.,* 2002).

NF-κB: Control of the inflammatory response and apoptosis are closely linked to NF-κB activation. NF-κB translocates to the nucleus where it activates pro-survival genes (Figure 12) including Bcl-XL, caspases inhibitors and c-Myb (BARKETT and GILMORE., 1999; LAUDER *et al.,* 2001). NF-κB negatively regulated by its inhibitor I-κBα which is degraded by activation of I-κBα kinase (IKK). Akt has been shown to regulate IKK activity by phosphorylating IKK at the threonin 23 residue. This activates IKK, which then targets I-κBα for proteasomal degradation (OZES *et al.,* 1999).

Cyclic AMP-response element-binding protein (CREB): Akt phosphorylates CREB on Ser133, leading to CREB activation and the promotion of cell survival through stimulation of the expression of the anti-apoptotic Mcl-1 (WANG *et al.,* 1999) and Bcl-2 (PUGAZHENTHI *et al.,* 2000). Finally, CREB phosphorylation and activation by Akt is also responsible for an increase in Akt expression, thus reinforcing Akt anti-apoptotic functions (REUSCH and KLEMM, 2002).

4.3.3 Regulation of cell growth and cell cycle progression by Akt

Akt promotes cell cycle progression and cell growth by phosphorylating different targets: glycogen synthase kinase-3 (GSK-3), mammalian target of rapamycin (mTOR), cyclin-dependent kinase inhibitors p21 and p27 (Figure 19).

In addition to upregulation of Mcl-1 protein (Figure 17), GSK-3 inhibition by Akt also prevents the phosphorylation of the cytoplasmic signaling molecule *β-catenin* by GSK-3, which prevents its degradation. β-catenin translocates to the nucleus and induces expression of *cyclin D1*, which induces cell cycle progression through the G1/S phase (DIEHL *et al.*, 1998).

Akt phosphorylates p21 and inhibits its antiproliferative effects by retaining it within the cytoplasm (ZHOU *et al.*, 2001). A similar mechanism has been described for p27 (LIANG *et al.*, 2002; SHIN *et al.*, 2002).

The *mammalian target of rapamycin (mTOR)* also known as mechanistic target of rapamycin is a serine/threonine protein kinase that regulates cell growth, cell motility, cell survival, protein synthesis. Activation of mTOR by Akt occurs through inactivation of the tuberous sclerosis complex (TSC) by phosphorylation. Unphosphorylated TSC complex blocks mTOR activation. This complex is disrupted by Akt-mediated phosphorylation. Thus, in the presence of mitogen stimulation of the PI3K/Akt pathway and sufficient nutrients, mTOR stimulates the translation of proteins such as cyclin D1 and c-myc required for the cell cycle progression from the G1 to the S phase (POTTER *et al.*, 2002; MANNING *et al.*, 2002; INOKI *et al.*, 2002).

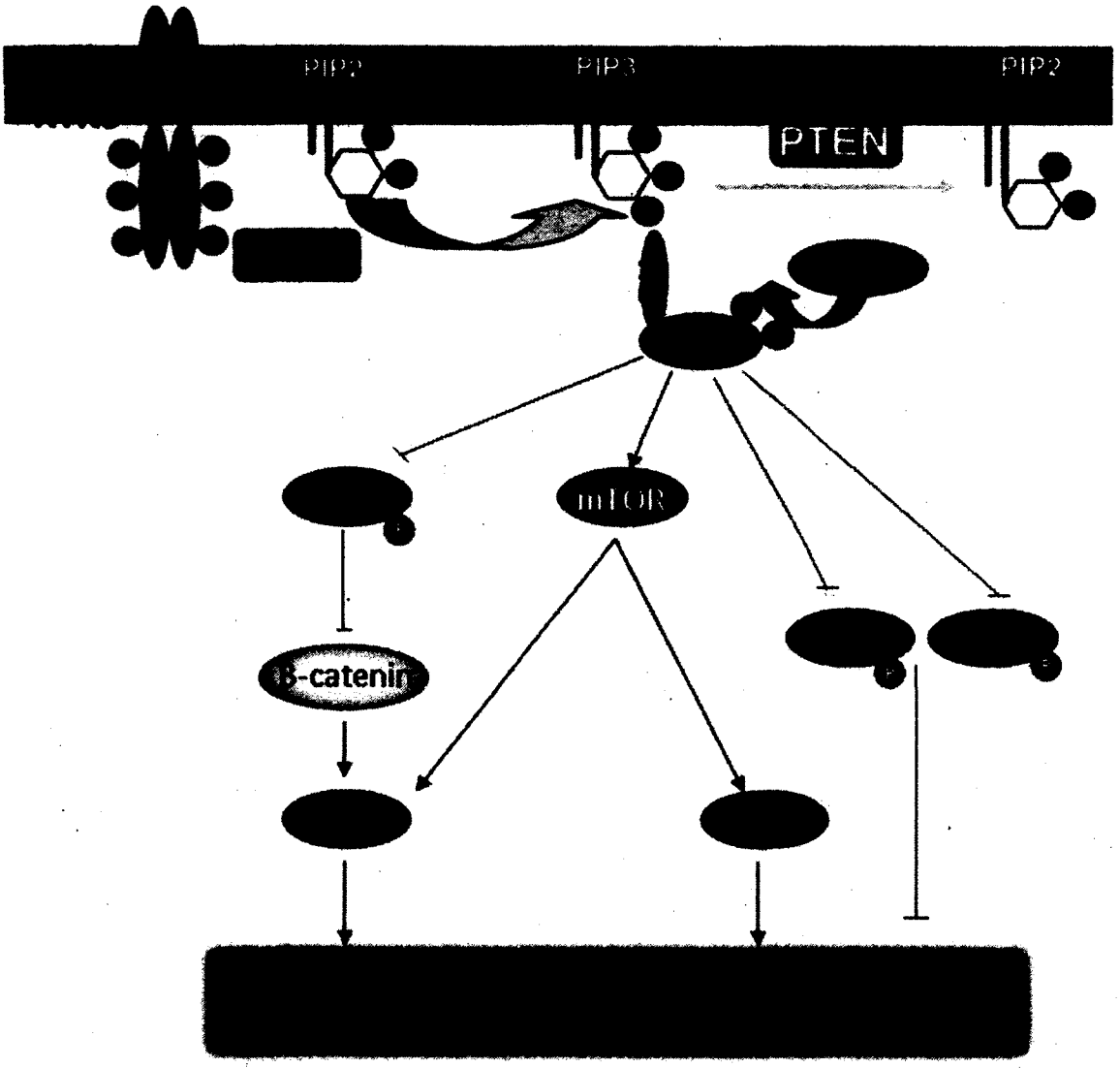

Figure 19. Regulation of cell cycle progression by Akt. Activated Akt promotes cell cycle progression through multiple mechanims, including direct phosphorylation of cyclin dependent kinase (CDK) inhibitors p21 and p27. Activation of mTOR by Akt stimulates the translation of proteins such as cyclin D1 and c-myc, both required for cell cycle progression from the G1 to the S phase. Cyclin D1 is further an indirect target of Akt through GSK-3-mediated transcription.

4.4 Inhibitors targeting PI3K/Akt pathway

Given the importance of the PI3K/Akt pathway, new inhibitors targeting this pathway are being developed. One of them is *Wortmannin* fungal metabolite that has shown to have antitumor activity *in vitro* and *in vivo* studies (POWIS *et al.*, 1994; SCHULTZ *et al.*, 1995). But a disadvantage of Wortmannin is its stability in an aqueous environment. Wortmannin is soluble in organic solvents, which limits its use in clinical trials. Stable water-soluble conjugates of Wortmannin are being developed to improve its pharmacological characteristics.

The flavonoid derivative, *LY294002*, is a competitive and reversible inhibitor of the ATP binding site of PI3K. Several studies have shown that LY294002 alone has antiproliferative and pro-apoptotic activities (CASAGRANDE *et al.*, 1998). LY294002 has a very short half-life and is insoluble in aqueous solutions (HENNESSY *et al.*, 2005).

The macrolide *rapamycin* and its derivates inhibit mTOR. All rapamycins under clinical development have antiproliferative activity as single agents (MUTHUKKUMAR *et al.*, 1995; SEUFFERLEIN and ROZENGURT, 1996).

5. Hypothesis and objectives of the project

Even though TRAIL represents a novel promising agent for the treatment of different types of cancer, resistance to this agent is common among EOC (LANE *et al.*, 2004). TRAIL is also known to induce survival and proliferation in TRAIL-resistant cancer cells (EHRHARDT *et al.*, 2003; LEVINA *et al.*, 2008). Understanding the mechanisms of resistance to TRAIL is essential in order to optimise the introduction of TRAIL into the clinic and to better understand if TRAIL treatment in patients that harbour tumor cells that are intrinsically resistant, shift the patient's treatment from beneficial to detrimental.

Several studies have provided insights on the mechanisms of acquired and de-novo resistance to TRAIL. Our laboratory demonstrated that that EOC ascites protected TRAIL-sensitive cells from TRAIL-induced apoptosis by activating Akt (LANE *et al.*, 2007). In addition, we demonstrated that cell detachment decreased Akt activity and sensitized TRAIL-resistant EOC cells to TRAIL (LANE *et al.*, 2007). But little is known about mechanisms of intrinsic resistance to TRAIL and the goal of this project was to better understand these mechanisms.

Based on the data described above we hypothesized that intrinsic resistance to TRAIL in EOC cell lines is associated with Akt activation. To investigate this hypothesis we selected two TRAIL-sensitive cell lines (CaOV3 and OVCAR3) and two TRAIL-resistant (SKOV3ip1 and COV2) cell lines (Figure 20). The basal level of activated (phosphorylated) Akt was significantly higher in TRAIL-resistant cell lines compare to TRAIL-sensitive.

Our first objective was to demonstrate that PI3K/Akt activation in EOC cells correlates with TRAIL resistance by investigating: 1-Akt activation and sensitivity to TRAIL among EOC primary samples, 2- by investigating how Akt overexpression in TRAIL-sensitive cell lines and Akt downregulation in TRAIL-resistant cell lines will affect sensitivity of these cells to TRAIL. The second objective was to identify the mechanisms by which Akt contributes to TRAIL resistance in EOC cells.

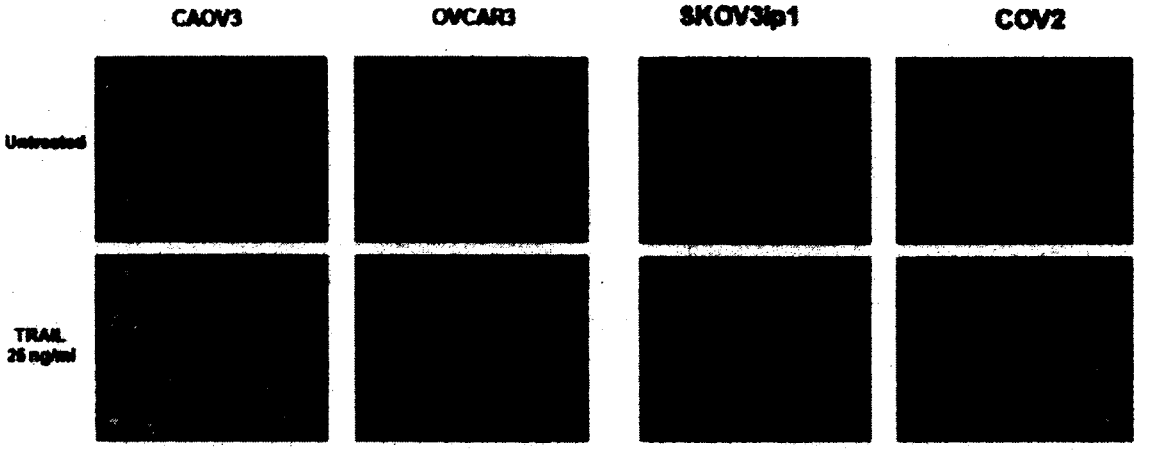

Figure 20. Effect of TRAIL on EOC cells. In the presence of TRAIL (cells were treated with 25 ng/ml TRAIL for 24h), sensitive CaOV3 and OVCAR3 cells were rounded and floating in the medium, suggesting massive cell death, whereas resistant SKOV3ip1 and COV2 cells form typical epithelial monolayer with only small proportion of rounded cells.

RÉSUMÉ DE L'ARTICLE

L'inhibition de l'expression de Bid par Akt augmente la résistance à l'apoptose induite par TRAIL dans les cellules de cancer ovarien.

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La voie de PI3K/Akt est souvent activée dans les cellules cancéreuses ovariennes (CEO), suggérant un rôle dans la pathogénèse. De plus, nous avons montré précédemment que les ascites provenant de patientes avec cancer ovarien épithélial induisaient une activation d'Akt chez la lignée de cancer ovarien CaOV3 sensible au TRAIL, provoquant une inhibition de l'apoptose médiée par TRAIL. Le but de l'étude présente est d'évaluer le rôle d'Akt dans la résistance intrinsèque au TRAIL, ce qui est fréquent dans les cellules de CEO. Nos résultats démontrent que l'activation d'Akt réduit la sensibilité au TRAIL des cellules de cancer ovarien. Les cellules résistantes au TRAIL, SKOV3ip1 et COV2, ont été sensibilisées à l'apoptose induite par le TRAIL avec des inhibiteurs de PI3K ou d'Akt, même si l'inhibition de la voie de signalisation PI3K/Akt n'interfère pas avec le recrutement et l'activation de la caspase-8 dans le complexe de signalisation induisant la mort. Réciproquement, la surexpression d'Akt1 dans les cellules sensibles au TRAIL, augmente la résistance au TRAIL. Même si le fait que l'activation de la caspase-8 induite par le TRAIL a été observée à la fois dans les lignées sensibles et dans les lignées résistantes, le clivage de Bid n'est observé que dans les cellules sensibles ou dans les cellules SKOV3ip1 traitées avec l'inhibiteur de PI3K, LY294002. Les cellules résistantes expriment des niveaux de Bid significativement plus faible que les cellules sensibles et l'activation de Bid régule négativement son expression. La déplétion de Bid au moyen de siRNA dans les cellules sensibles OVCAR3 est associée avec une diminution de l'apoptose médiée par TRAIL. Un blocage simultané de la voie d'Akt augmentait d'avantage l'apoptose induite par TRAIL. Donc, Akt agit en amont de la mitochondrie et inhibe

l'apoptose induite par TRAIL en diminuant les niveaux de la protéine Bid et possiblement en inhibant son clivage.

ORIGINAL ARTICLE

The inhibition of Bid expression by Akt leads to resistance to TRAIL-induced apoptosis in ovarian cancer cells

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ABSTRACT

Epithelial ovarian cancer (EOC) cells often show increased activity of the PI3K/Akt pathway. In addition, we have previously shown that EOC ascites induce Akt activation in the tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-sensitive EOC cell line, CaOV3, leading to TRAIL-mediated apoptosis inhibition. In this study, we investigated the role of Akt in intrinsic resistance to TRAIL, which is common in EOC cells. We report that Akt activation reduces the sensitivity of EOC cells to TRAIL. TRAIL-resistant SKOV3ip1 and COV2 cells were sensitized to TRAIL-induced apoptosis by PI3K or Akt inhibitors although inhibition of PI3K/Akt signaling pathway did not interfere with the recruitment and processing of caspase-8 to the death-inducing signaling complex. Conversely, overexpression of Akt1 in TRAIL-sensitive cells promoted resistance to TRAIL. Although the fact that TRAIL-induced caspase-8 activation was observed in both sensitive and resistant cell lines, Bid cleavage occurred only in sensitive cells or in SKOV3ip1 cells treated with LY294002. Bid expression was low in resistant cells and Akt activation downregulated its expression. Depletion of Bid by siRNA in OVCAR3 cells was associated with a decrease in TRAIL-mediated apoptosis. Overexpression of Bid only in SKOV3ip1 cells enhanced TRAIL-induced apoptosis. Simultaneous blockade of Akt pathway further increased TRAIL-induced apoptosis. Thus, Akt acts upstream of mitochondria and inhibits TRAIL-induced apoptosis by decreasing Bid protein levels and possibly inhibiting its cleavage.

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Keywords: death receptors; ovarian carcinoma; resistance; PI3K/Akt pathway; TRAIL; Bid

INTRODUCTION

Resistance to chemotherapy is a major problem in epithelial ovarian cancer (EOC) treatment. Despite initial high response rate in patients with advanced EOC, most patients relapse with tumor that acquires resistance to chemotherapy (Yap *et al.,* 2009). The tumor necrosis factor related-apoptosis-inducing ligand (TRAIL) holds great promise as an anti-cancer therapy because of its selective apoptosis-inducing action on tumor cells vs. normal cells (Newsom-Davis *et al.,* 2009). TRAIL-based therapies are now in phase I/II clinical trials (http://www.clinicaltrials.gov). TRAIL resistance in tumor cells, including EOC, may limit its therapeutic use (LeBlanc *et al.,* 2002; Lane *et al.,* 2004; Zhang and Fang, 2005). On binding to its receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) TRAIL induces the formation of the death-inducing signaling complex (DISC) by recruiting Fas-associated death domain. Fas-associated death domain through its death effector domain recruits pro-caspases-8/10, which assemble into the DISC (Kischkel *et al.,* 1995; Bodmer *et al.,* 2000). When recruited to the DISC, pro-caspase-8 is activated through proteolytic cleavage, resulting in active caspase-8 that may directly cleave downstream effector caspases (caspase-3, -6 and -7) leading to apoptosis. Alternatively, caspase-8 cleaves the BH3 domain-containing protein Bid, to generate a pro-apoptotic truncated form (tBid), which translocates to the mitochondrial membrane and triggers the intrinsic apoptosis pathway.

Although there are a number of means by which the association between TRAIL signaling cascade and the apoptotic machinery could be altered in TRAIL-resistant EOC cells, the phosphatidylinositol-3-kinase PI3K/Akt pathway is of particular interest because it is an important determinant of oncogenic transformation in EOC. The PI3K/Akt pathway is activated in a significant number of EOC cells (~70%) because of amplification/ overexpression/mutation (Bast *et al.,* 2009). Activation of the PI3K/Akt pathway was shown to have a role in protecting EOC cells from chemotherapy-induced apoptosis (Page *et al.,* 2000; Hu *et al.,* 2002; Yang *et al.,* 2006). Drugs and death receptor ligands rely on activation of apoptotic signaling pathways to destroy tumor cells. Indeed, the resistance to chemotherapy is because of the failure of tumor cells to undergo apoptosis. As shown by us and others, death receptor-mediated apoptosis is deficient in nearly 50% of ovarian cancer

cell lines and primary tumors (Lane *et al.,* 2004). Consequently, to fully exploit the potential of TRAIL, the problem of TRAIL resistance in EOC cells must be first understood.

Akt activation was shown to protect against TRAIL-induced apoptosis in several cell types, including melanomas and prostate, ovarian and non-small cell lung cancers (Chen *et al.,* 2001; Nesterov *et al.,* 2001; Kandasamy and Srivastava, 2002; Larribere *et al.,* 2004; Kim and Lee, 2007; Lane *et al.,* 2007). However, there are conflicting reports about the mechanisms by which Akt exerts its protective effect. In non-small cell lung and prostate cancer, Akt activation inhibited TRAIL-induced apoptosis by interfering with Bid cleavage by means of an unknown mechanism (Chen *et al.,* 2001; Nesterov *et al.,* 2001; Kandasamy and Srivastava, 2002). Inhibition of PI3K or Akt completely blocked the anti-apoptotic effect of stem cell factor on TRAIL-induced apoptosis in melanoma cells (Larribere *et al.,* 2004). FLIP did not appear to be involved but the precise mechanism was unclear. In TRAIL-sensitive EOC cells, inhibition of PI3K and Akt substantially inhibited the pro-survival effect of EOC ascites by a mechanism that involved upregulation of c-FLIPs (Lane *et al.,* 2007).

Previous studies have shown that EOC cell susceptibility to TRAIL cannot be explained by the levels of TRAIL receptors (Cuello *et al.,* 2001; Siervo-Sassi *et al.,* 2003; Lane *et al.,* 2004). TRAIL induces mitochondria-dependent apoptosis in EOC, a characteristic of the type II cells (Cuello *et al.,* 2004). Bid has a central role in type II cells by connecting the mitochondria pathway of apoptosis to the extrinsic pathway (Newsom-Davis *et al.,* 2009). Akt regulates the activity of Bad, Bax and X-linked inhibitor of apoptosis (XIAP) to promote survival (Datta *et al.,* 1997; del Peso *et al.,* 1997; Kandel and Hay, 1999; Dan *et al.,* 2004). Akt may also upregulate the expression of anti-apoptotic proteins such as Bcl-2 (Du and Montminy, 1998). However, a recent study has shown that Akt activation does not affect the expression of Bcl-2, Bcl-XL and Bax (Lane *et al.,* 2007). Thus, the mechanisms by which Akt confers protection and its action on the mitochondrial pathway in TRAIL-induced apoptosis remains to be fully understood in EOC cells.

In this study, we have examined the role of Akt and the mechanism of intrinsic TRAIL resistance in ovarian cancer cells. We report for the first time that Akt activation inhibits TRAIL-induced apoptosis by decreasing Bid expression. Furthermore, Bid depletion by RNA interference led to a decrease of TRAIL-induced apoptosis. These results show that Akt acts upstream of the mitochondria at the Bid level to inhibit TRAIL-induced cell death in EOC cells.

RESULTS

TRAIL resistance is associated with Akt activation in EOC cells

We have previously shown that ascites-mediated Akt activation protects CaOV3 cells against TRAIL-induced apoptosis (Lane *et al.,* 2007). In addition, we showed that cell detachment decreased Akt activity and sensitized SKOV3ip1 and COV2 EOC cells to TRAIL (Lane *et al.,* 2008). These data suggest that Akt may be an important regulator of TRAIL-induced apoptosis. In this study, we investigated whether Akt is indeed a mediator of TRAIL resistance. For our studies, four EOC cell lines were used. The cell lines were subdivided into sensitive (<20% cell viability; CaOV3 and OVCAR3 cells) and resistant (80% cell viability; COV2 and SKOV3ip1 cells) to TRAIL-induced cell death (Figure 1a). In sensitive cells, TRAIL treatment decreased the number of colonies by 10-fold whereas the number of colonies remained mostly unchanged in resistant cells (Figure 1b). TRAIL-induced apoptosis was significantly greater in sensitive cells when compared with COV2 and SKOV3ip1 cells as measured by oligosomal DNA fragmentation (Figure 1c) and the percentage of hypodiploid cells (Figure 1d).

We examined the basal level of phospho-Akt in our panel of cell lines. As shown in Figure 1e, there was a positive correlation between high levels of Akt phosphorylation and resistance to TRAIL. Interestingly, however, there was no correlation between levels of c-FLIPs or c-$FLIP_L$ proteins, which inhibit caspase-8 activation at the DISC (Kruger *et al.,* 2001; Panka *et al.,* 2001; Panner *et al.,* 2005), and the sensitivity of EOC cell lines to TRAIL-induced apoptosis.

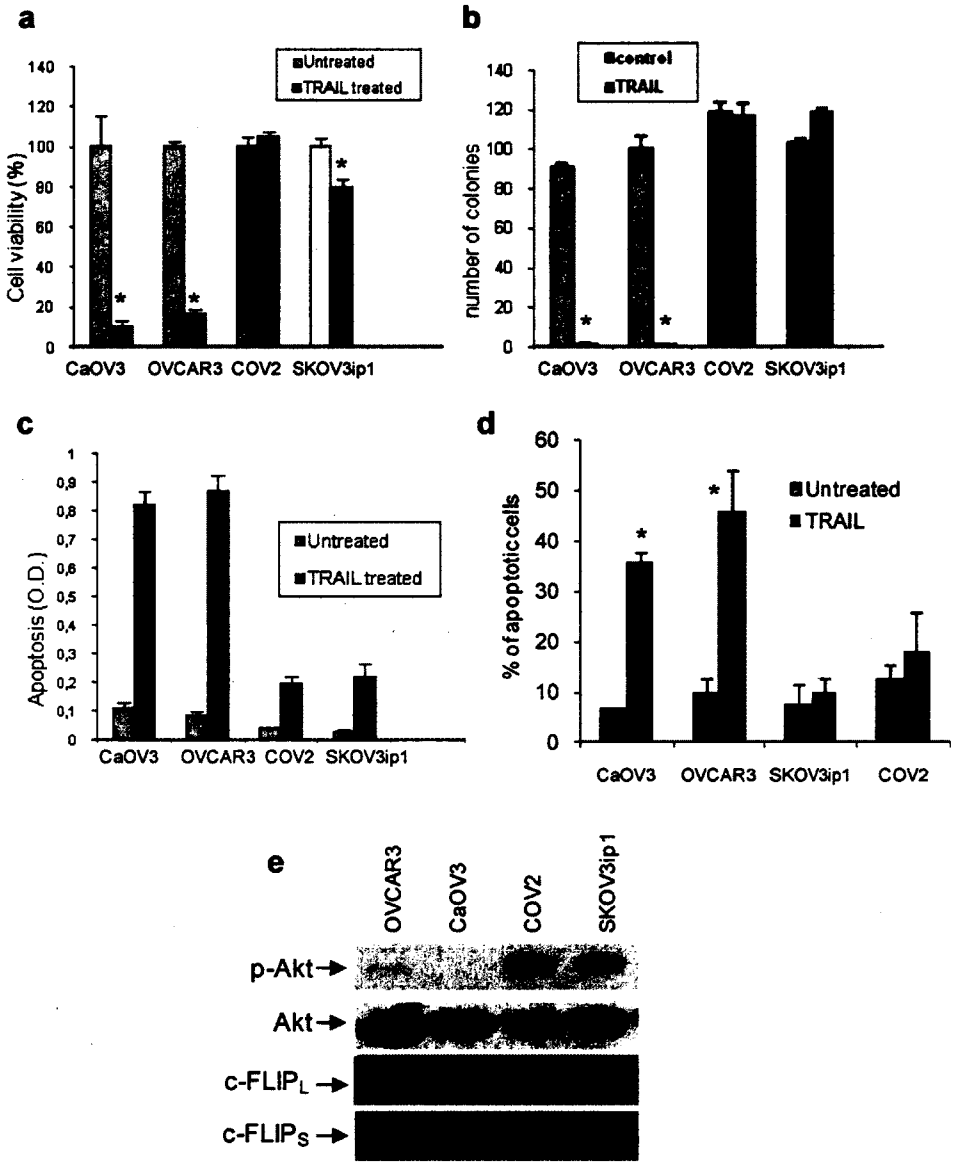


Figure 1 *Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance correlates with Akt activation in ovarian cancer cells.* Cell viability was assessed in four ovarian cancer cell lines by a short-term assay where cells were incubated with TRAIL (100 ng/ml) for 48 h and viability was assessed by XTT assay **(a),** and by a long-term assay where cells were treated with TRAIL (100 ng/ml) or left untreated (control) for 24 h and colonies were counted after 14 days **(b).** Apoptosis was measured by determining oligosomal DNA fragmentation **(c)** and the percentage of apoptosis **(d)** was measured as percentage of hypodiploid cells assessed by flow cytometry analysis during exposure to TRAIL (100 ng/ml) for 24 h. Data shown are means ± s.e.m. derived from three independent experiments. **(e)** Expression/phosphorylation of Akt, $FLIP_L$ and $FLIP_S$ were analyzed by western blotting with appropriate antibodies. Representative immunoblot from three independent experiments. *$P<0.001$.

Overexpression of Akt1 inhibits TRAIL-induced apoptosis in CaOV3 cells

We determined whether Akt1 overexpression would exert a protective effect against TRAIL-induced apoptosis. CaOV3 cells overexpressing Akt1 (CaOV3-Akt-1 (wt)) or vector-transfected cells (CaOV3 (vector)) were validated by immunoblot (Figure 2a). Of note, the ectopic expression of Akt1 did not affect the protein levels of $FLIP_L$ and $FLIP_S$ (data not shown). CaOV3-Akt-1 (wt) cells were significantly ($P<0.001$) more resistant to TRAIL compared with CaOV3 (vector) cells as indicated by a dose-response curve (Figure 2b) and colony formation after TRAIL exposure (20 ng/ml) (Figure 2c). However, the resistance of CaOV3-Akt-1 (wt) cells can be overcome by higher concentrations of TRAIL (Figure 2b). We believe that this may be related to relatively low levels of pAkt in CaOV3-Akt-1 (wt) cells despite high expression of total Akt. The level of cell death for CaOV3 (vector) cells was comparable to that of the parental CaOV3 cells (data not shown). Oligosomal DNA fragmentation and the percentage of hypodiploid cells were used to measure apoptosis. Akt1 overexpression decreased TRAIL-induced DNA fragmentation (Figure 2d) and the percentage of apoptosis (Figure 2e) to a level equal to untreated cells, suggesting that TRAIL-induced apoptosis is inhibited by expression/activation of Akt. Interestingly, despite showing resistance to TRAIL, CaOV3-Akt-1 (wt) cells showed similar levels of caspase-8 activity to control CaOV3 (vector) cells after TRAIL treatment (Figure 2f). However, caspase-3 activity was significantly lower in Akt1-expressing CaOV3 cells as compared with control cells. These data suggest that Akt confers resistance to TRAIL-induced apoptosis by interfering downstream of caspase-8.

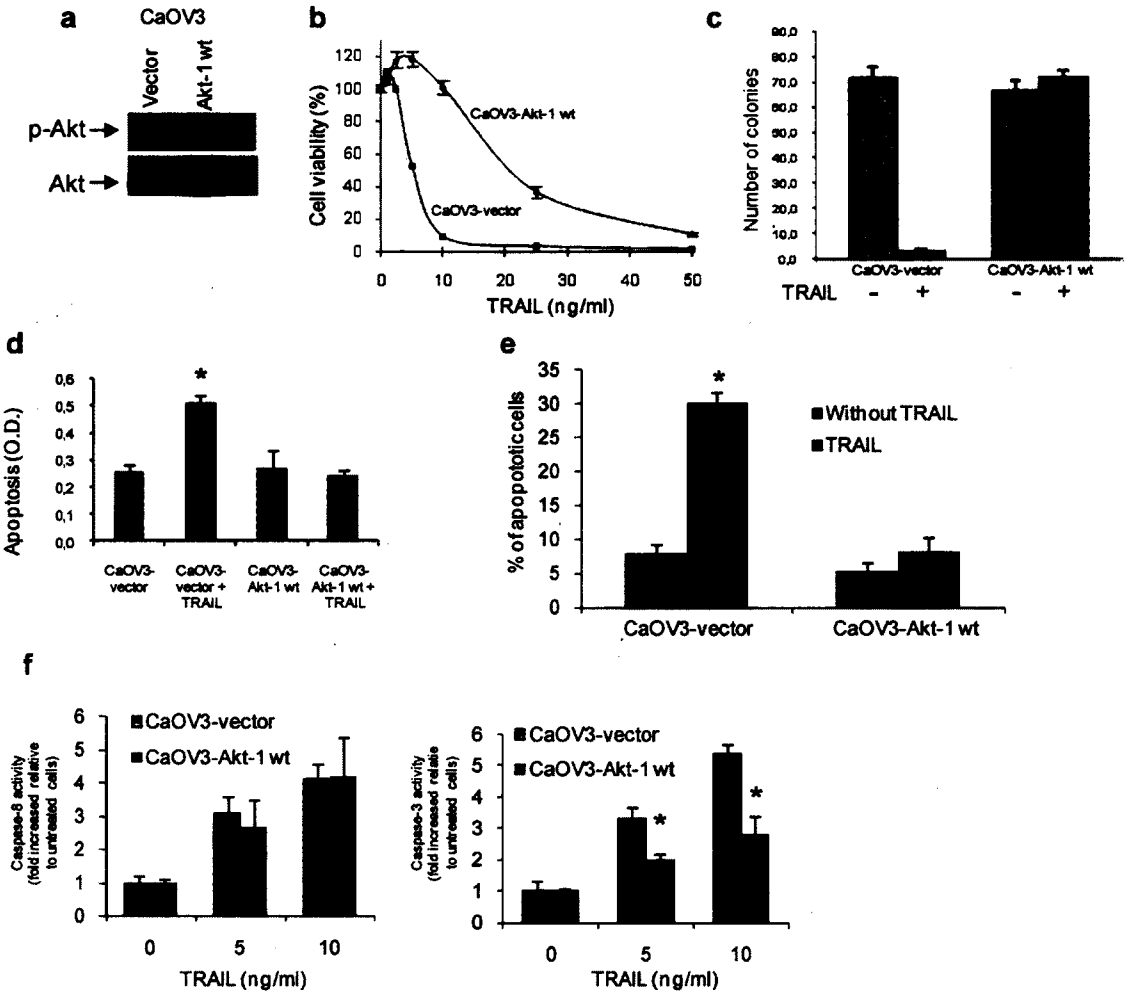


Figure 2 *Overexpression/phosphorylation of Akt inhibits TRAIL-induced apoptosis in CaOV3 cells.* TRAIL-sensitive CaOV3 cells were stably transfected with empty vector (CaVO3 (vector)) or with Akt1-encoding construct (CaOV3-Akt-1 (wt)). **(a)** After selection, cells were analyzed for Akt expression and phosphorylation by immunoblot. Cell viability was assessed by short-term XTT assay in the presence of increasing concentration of TRAIL **(b)** or by long-term assay where cells were left untreated or exposed to TRAIL (20 ng/ml) for 24 h. After 14 days colonies were counted **(c)**. Apoptosis was measured by determining oligosomal DNA fragmentation **(d)** and the percentage of apoptosis **(e)** was measured as percentage of hypodiploid cells assessed by flow cytometry analysis during exposure to TRAIL (20 ng/ml) for 24 h. Data shown are means ± s.e.m. derived from three independent experiments. **(f)** Caspase-8 and caspase-3 activity were measured using fluorogenic substrates in CaOV3-vector and CaOV3-Akt-1 wt cells treated with TRAIL (0 to 10 ng/ml). Data shown are means ± s.e.m. derived from three independent experiments. *P<0.001.

PI3K and Akt inhibitors enhance TRAIL-induced apoptosis in resistant cells

To further confirm the role of Akt, we assessed whether PI3K inhibitor LY294002 or Akt inhibitor blocked Akt activation and enhanced TRAIL-sensitivity in resistant cells. Treatment of SKOV3ip1 and COV2 cells with LY294002 (5 μM) for 60 min strongly blocked Akt phosphorylation (Figure 3a). Although we did not find any correlation between c-FLIPs or c-FLIPL protein levels and TRAIL sensitivity (Figure 1e), we assessed the effect of LY294002 on c-FLIPs or c-$FLIP_L$ expression to ensure that Akt activity does not affect their expression. Figure 3b shows no effect of PI3K inhibition on c-FLIPs or c-$FLIP_L$ protein levels in SKOV3ip1 cells. Similarly, the levels of pAkt and total Akt were not affected by TRAIL in SKOV3ip1 cells (Figure 3c). Pre-incubation of SKOV3ip1 or COV2 cells with LY294002 (5 μM) or Akt inhibitor (10 μM) sensitized these cells to TRAIL-induced apoptosis as shown by increased oligosomal DNA fragmentation (Figure 3d) and increased rounded dead cells (Figure 3e) compared with controls.

Akt activation in human primary samples of EOC correlated with decreased TRAIL sensitivity

To determine whether Akt activation is correlated with decreased TRAIL sensitivity in clinical samples of EOC, we obtained primary samples of tumor cells from women having serous ovarian carcinoma. The sensitivity of primary ovarian tumors cells to TRAIL was determined by XTT assay as described previously (Lane *et al.,* 2004), and expressed as IC_{50}. The relative expression of pAkt in primary samples was assessed by western blotting, measured by densitometric quantification and expressed as fold increased relative to pAkt levels in CaOV3 cells. pAkt levels in primary tumor samples correlated well and positively with increased TRAIL IC_{50}, indicating that Akt activation confers resistance to TRAIL in vivo ($n=12$, Pearson's $r=0.950$; $P<0.0001$).

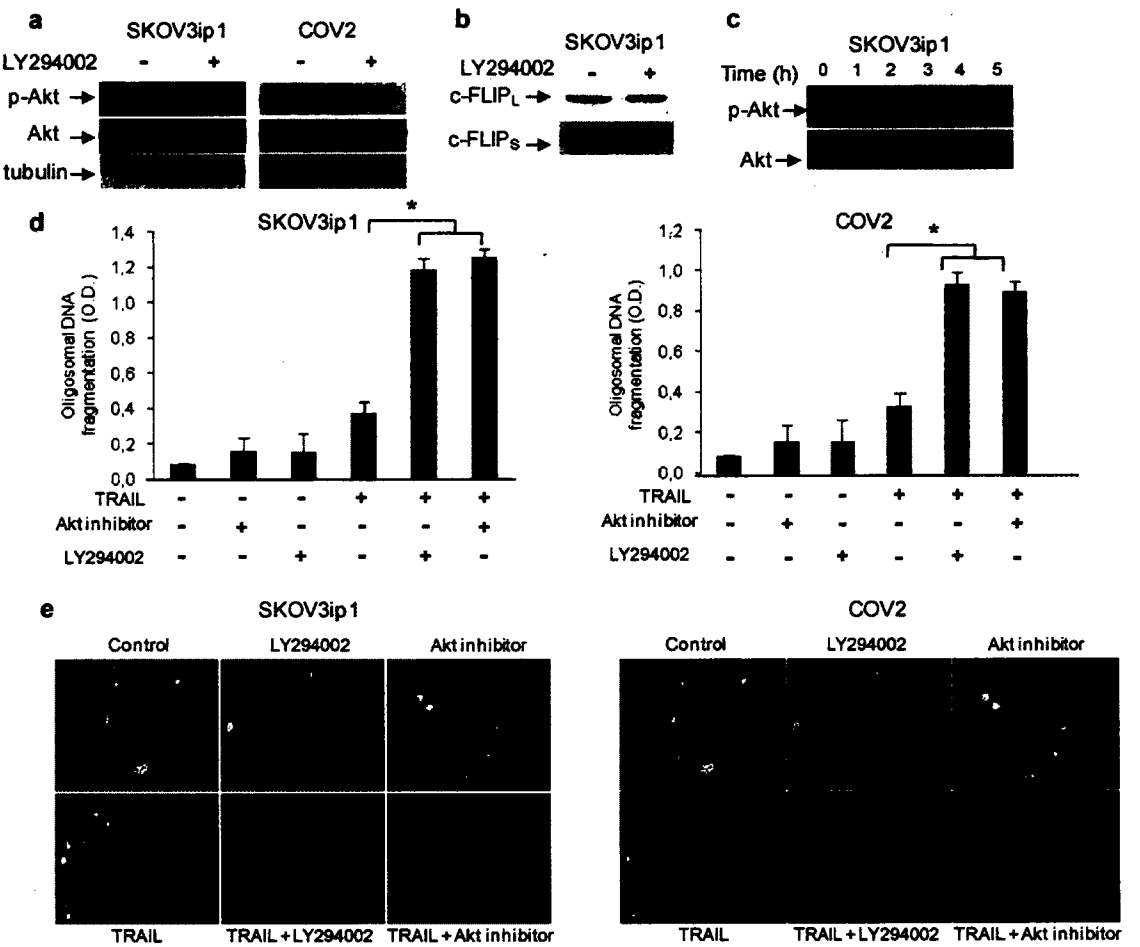


Figure 3 *Inhibition of the PI3K/Akt pathway sensitizes ovarian cancer cells to TRAIL-induced apoptosis.* TRAIL-resistant SKOV3ip1 and COV2 cells were incubated with LY294002 (5 μM) for 60 min, after which immunoblot analysis was performed to determine **(a)** Akt phosphorylation and **(b)** $FLIP_L$ and $FLIP_S$ expression. **(c)** SKOV3ip1 cells were treated with TRAIL (100 ng/ml) and Akt phosphorylation was monitored for up to 5 h. Resistant cells were incubated with LY294002 (5 μM) or Akt inhibitor (10 μM) for 60 min, after which TRAIL (100 ng/ml) was added for 48 h. Apoptosis was determined by measuring oligosomal DNA fragmentation **(d)** and visualizing cells by optical microscopy **(e)**. Data shown are representative of three independent experiments. Values are the means ± s.e.m. *$P<0.001$.

TRAIL resistance is downstream of caspase-8 but upstream of mitochondria

Analysis of caspase activation indicated that the pro-caspases were processed and cleaved fragments of caspase-8 (p43/41, p18) and caspase-9 (p35) were generated in TRAIL-sensitive OVCAR3 cells (Figure 4a). The generation of active caspase-8 and caspase-3 fragments was confirmed by measuring the cleavage of caspase-8 and caspase-3 substrates in OVCAR3 cells (Figure 4b). In contrast, only partially processed caspase-8 p43/41 fragments were detected in SKOV3ip1 and neither caspase-8 nor caspase-9 mature fragments were detected. The measurement of caspase-8 activity however, showed substantial caspase-8 substrate cleavage in SKOV3ip1 cells although to a lesser extend relative to OVCAR3 cells (Figure 4b). This indicated that caspase-8 is activated in SKOV3ip1 but probably at a level which is too low to be detected by an immunoblot. Caspase-3 activity was not, however, detected in SKOV3ip1 cells (Figure 4b). As binding of TRAIL to its receptors causes recruitment of pro-caspase-8 at part of the DISC, resulting in activation of caspase-8, we immunoprecipitated the DISC in cells treated with FLAG-tagged TRAIL. As shown in Figure 4c, pro-caspase-8 was recruited and cleaved at the DISC in a time-dependent manner in both sensitive and resistant cells. Of note, recruitment and processing of pro-caspase-8 at the DISC was not affected by LY294002 (Figure 4d). Similarly, caspase-8 activity in total cell lysate of SKOV3ip1 cells was not inhibited by LY294002 (Figure 4e) indicating that Akt blockade occurs downstream of caspase-8.

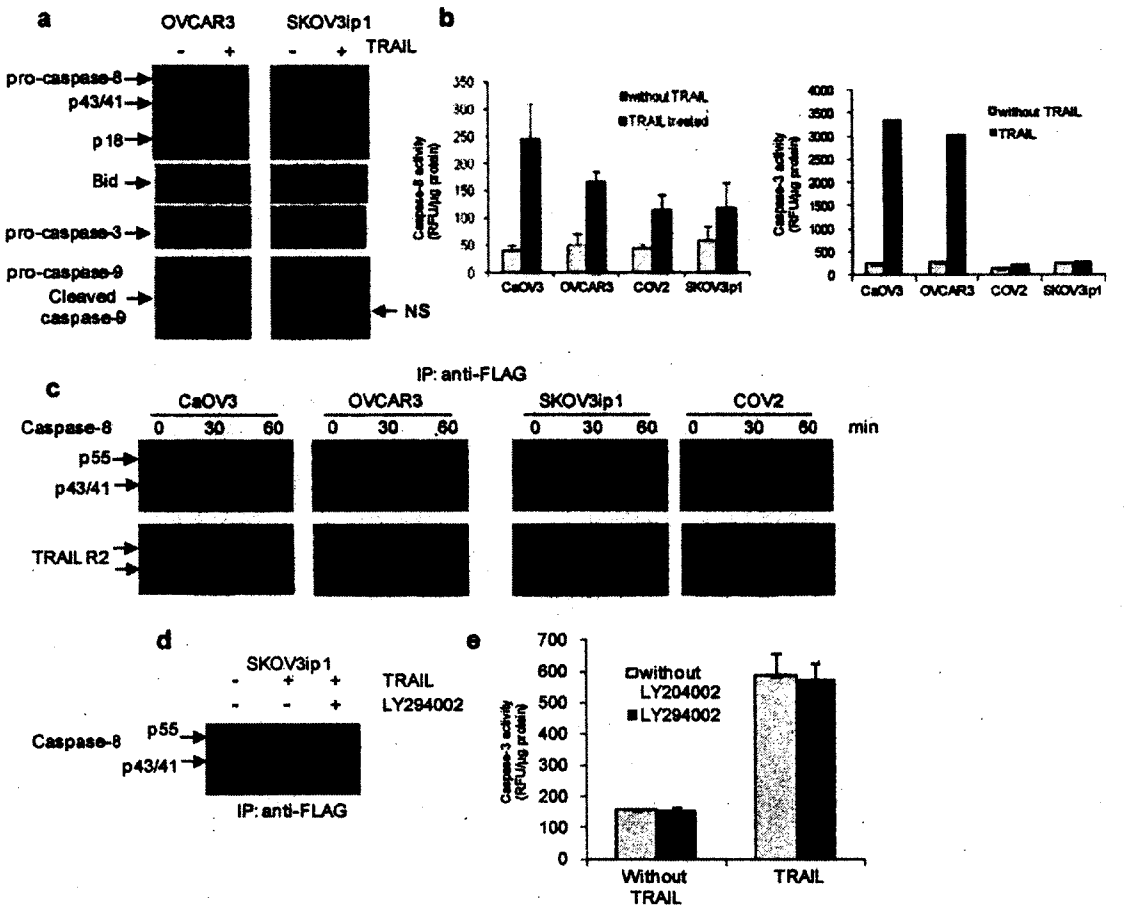


Figure 4 *Blockade of the TRAIL signaling cascade is located downstream of caspase-8 in resistant cells.* **(a)** Caspase cleavage during TRAIL treatment (100 ng/ml) was assessed by immunoblot analysis after 24 h in TRAIL-sensitive CaOV3 and TRAIL-resistant SKOV3ip1. NS, nonspecific band. **(b)** Caspase-8 and caspase-3 activity in sensitive and resistant epithelial ovarian cancer (EOC cells) were measured by the cleavage of fluorogenic substrate IETD-AFC and DEDV-AFC after TRAIL treatment (100 ng/ml) for 24 h. **(c)** TRAIL receptors were immunoprecipitated using Flag-tagged recombinant TRAIL and anti-Flag M2 antibody at various times after TRAIL treatment. Co-immunoprecipitated DR5 (doublet) and caspase-8 (pro-caspase-8, p55 and cleavage fragments, p43/41) were revealed by immunoblotting. **(d)** SKOV3ip1 cells were incubated for 1 h with LY294002 (5 μM), after which caspase-8 was co-immunoprecipitated as described above following Flag-tagged TRAIL treatment for 1 h. **(e)** Caspase-8 activity was measured in SKOV3ip1 cells treated with LY294002 alone or in combination with TRAIL (100 ng/ml) for 24 h.

Mitochondrial activation is an essential event for efficient TRAIL-induced apoptosis in EOC cells (Cuello *et al.*, 2004). We, therefore, examined the effect of TRAIL on mitochondrial outer membrane permeabilization and cytochrome c release in OVCAR3 and SKOV3ip1 cells. Mitochondrial outer membrane permeabilization was assessed by the uptake of a lipophilic cationic dye where red fluorescence represents intact mitochondria membrane and green fluorescence represents apoptotic mitochondria. Treatment of OVCAR3 cells with TRAIL, increased the number of green labeled mitochondria (Figure 5a) and consequently the percentage of apoptotic mitochondria as compared with SKOV3ip1 cells (Figure 5b). Heavy membrane, enriched in mitochondria and cytosolic fractions, were isolated from OVCAR3 and SKOV3ip1 cells, after treatment with TRAIL. Cytochrome c was detected in the cytosol of OVCAR3 cells as early as 2 h after TRAIL treatment whereas cytochrome c was not detected in SKOV3ip1 even after 8 h of TRAIL treatment (Figure 5c). These results suggest the mitochondrial cell death pathway is inhibited in resistant cells.

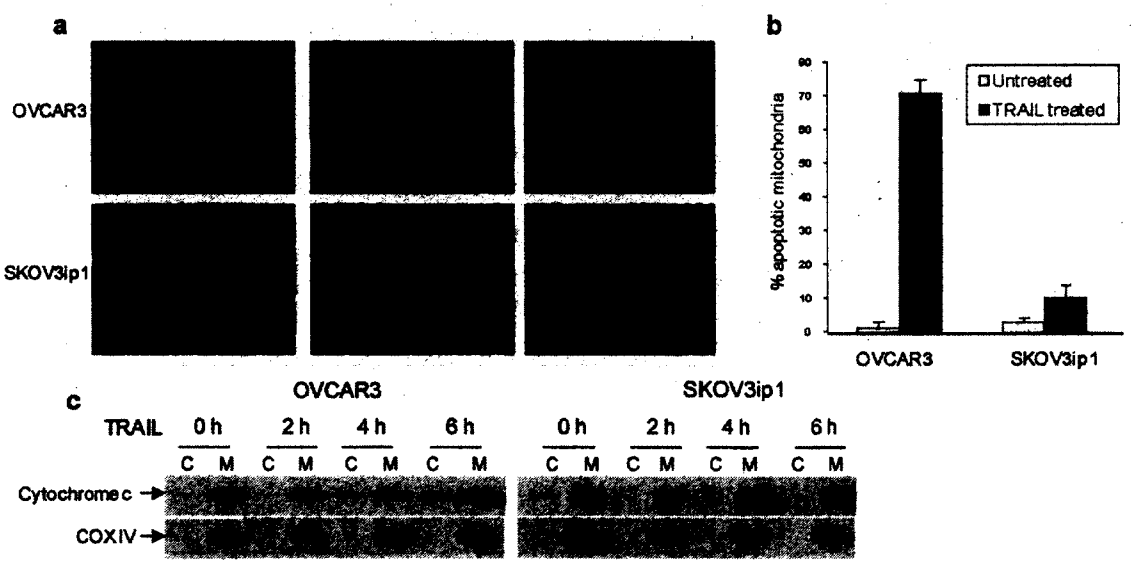


Figure 5 *Lack of mitochondrial activation in TRAIL-resistant cells.* **(a)** OVCAR3 and SKOV3ip1 cells were cultured for 24 h without TRAIL and the mitochondrial membrane integrity was assessed using MitoLight apoptotic detection kit staining. In treated cells, fresh culture medium containing TRAIL (100 ng/ml) was added for 5 h before subjected to MitoLight apoptotic detection kit staining. Only TRAIL-treated cells are shown. The red fluorescence (left panels) represents dimeric dye that has accumulated in the intact mitochondria membrane representing non-apoptotic cells. The green fluorescence (middle

panels) represents cytoplasmic pools of monomeric-lipophilic-cationic dye indicating the the lack of ability of mitochondria to concentrate the dye and consequently shows apoptotic cells. Right panels represent overlays of left and middle panels. **(b)** Percentage of apoptotic mitochondria in OVCAR3 and SKOV3ip1 cells during TRAIL treatment. **(c)** OVCAR3 and SKOV3ip1 cells were treated with TRAIL for different times and levels of cytochrome c in cytosolic (C) and membrane (M) fractions were determined by western blot. COX IV was used as a mitochondrial marker and loading control.

tBid is not detected in resistant cells

TRAIL-induced Bid cleavage generates a truncated form of Bid (tBid) that promotes the insertion of Bax into the mitochondrial outer membrane. As shown in Figure 6a, TRAIL (100 ng/ml) treatment resulted in a reduction in full-length Bid and the appearance of tBid overtime in sensitive cells but not in resistant cells suggesting that Akt interfere with caspase-8-mediated Bid cleavage. To further support this observation, SKOV3ip1 and COV2 cells were treated with TRAIL (100 ng/ml) in the presence or absence of LY294002 (5 μM). When TRAIL was combined with LY294002, there was a reduction of full-length Bid, but we did not detect tBid presumably, because the levels of tBid are too low to be detected by immunoblot (Figure 6b). Overexpression of Akt1 in CaOV3 cells prevented TRAIL-induced Bid cleavage (Figure 6c). These results suggest that Akt inhibits TRAIL induced activation of the mitochondrial cell death pathway by preventing the accumulation of tBid at levels sufficient to induce apoptosis.

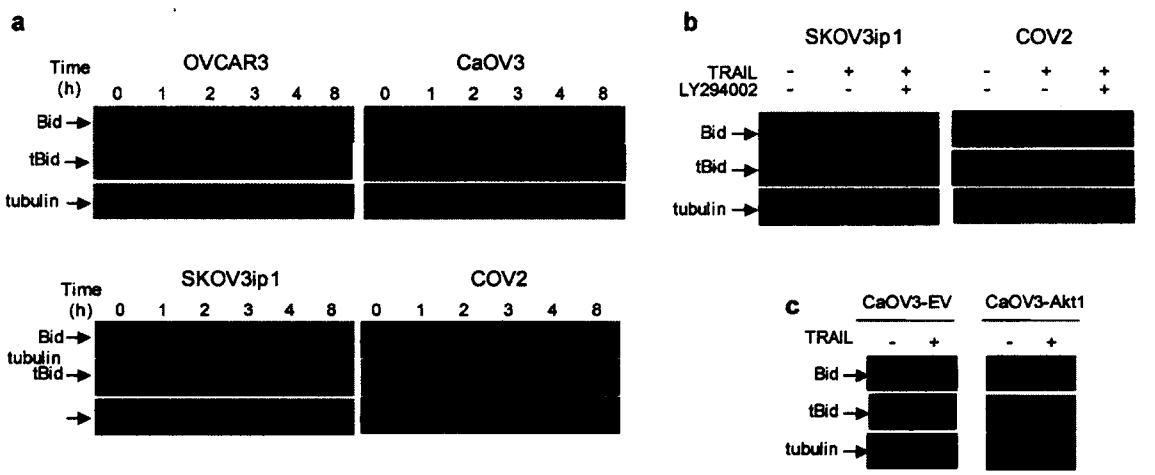


Figure 6 *Effect of Akt on Bid cleavage.* **(a)** Immunoblot analysis for the assessment of Bid cleavage. Sensitive and resistant cells were treated with TRAIL (100 ng/ml) for various times and Bid cleavage was determined by the decrease of full-length Bid protein and the appearance of tBid on western blot using anti-Bid antibodies. **(b)** TRAIL-resistant SKOV3ip1 and COV2 cells incubated with LY294002 (5 μM) or left untreated for 1 h before adding TRAIL for 8 h. Cell lysates were analyzed by western blotting using the indicated antibodies. **(c)** CaOV3 cells expressing the empty vector (CaOV3-EV) or Akt1 (CaOV3-Akt1) were treated with TRAIL for 8 h. Cell lysates were analyzed as described above. Tubulin was used to ensure equal loading.

Akt activation decreases Bid protein levels

We compared levels of Bid protein in our TRAIL-sensitive and -resistant cell lines. The expression of Bid was lower in resistant cells, both, at the protein and transcriptional levels (Figures 7a and e). In contrast, the expression of Bcl-2, Bcl-XL, Mcl-1 and Bax did not correlate with TRAIL sensitivity as shown by immunoblot (Supplementary Figure S1 online). We next examined Bid expression in CaOV3-EV and CaOV3-Akt1 cells. The levels of Bid protein and mRNA were lower in Akt1 overexpressing cells relative to CaOV3-vector cells (Figures 7b and f). When Akt activation was inhibited by a PI3K inhibitor, LY294002, in TRAIL-resistant cells, Bid expression was increased (Figure 7c). To further evaluate whether Akt inhibits Bid expression at the transcriptional level, we carried out Bid qRT–PCR at 2 and 6 h after exposure of CaOV3 cells to EOC ascites, which induce Akt activation within 30 min (Lane *et al.*, 2010). As shown in Figure 7d, there was a decrease in Bid mRNA expression at 2 and 6 h. Taken together, these data suggest that Akt inhibits TRAIL-induced apoptosis by decreasing the expression of Bid.

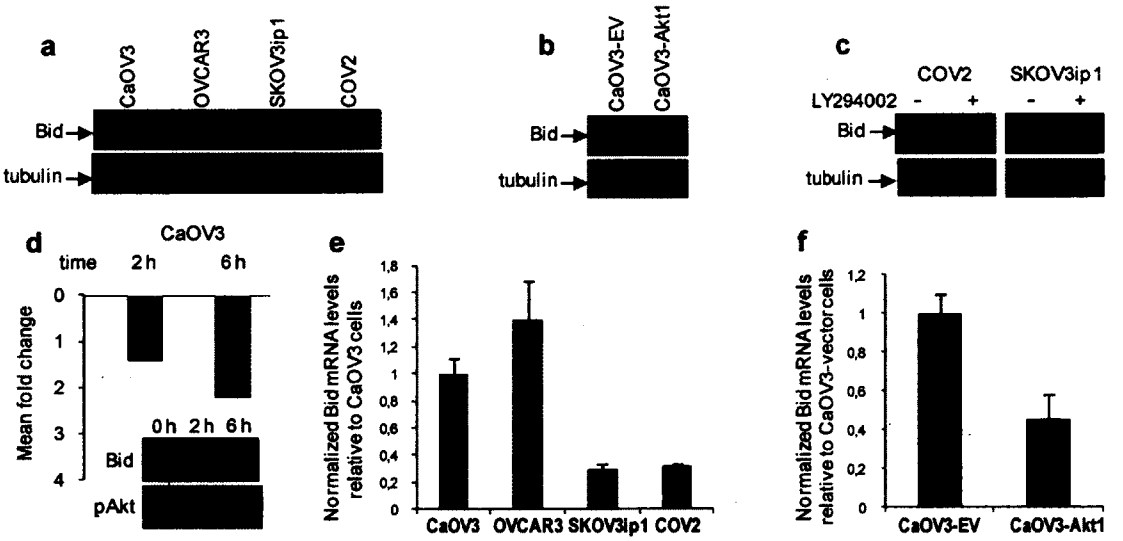
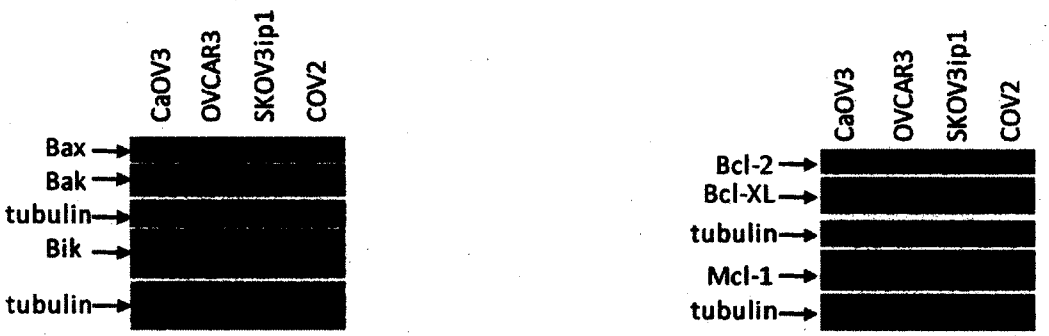


Figure 7 *Akt downregulates Bid expression.* **(a)** Western blot analysis of Bid expression in TRAIL-sensitive and -resistant cells. **(b)** Bid expression in CaOV3 cells expressing the empty vector (CaOV3-EV) or Akt1 (CaOV3-Akt1) assessed by western blot using anti-Bid

antibodies. (c) SKOV3ip1 and COV2 were treated with LY294002 (5 μM) or incubated in standard medium for 1 h and Bid expression was assessed by western blot. Tubulin was used to ensure equal loading. (d) CaOV3 cells were exposed to EOC COV2 ascites (10% v/v) to induce Akt phosphorylation at 2 and 6 h later, qRT–PCR analysis was performed for detecting Bid mRNA as described in Material and methods. Histogram represents mean-fold change of Bid mRNA levels relative to cells which were not exposed to ascites. Bid expression and Akt phosphorylation were also assessed by western blot at 2 and 6 h to confirm that the decreased Bid at the protein level correlates with the phosphorylation of Akt. (e) Expression of Bid mRNA by qRT–PCR in our sensitive and resistant cell lines. Histogram represents of mean of normalized Bid mRNA and expressed relative to Bid mRNA levels in CaOV3 cells. (f) Histogram of mean of normalized Bid mRNA in CaOV3 (vector) and CaOV3-Akt-1 (wt) cells. Bid mRNA levels are expressed relative to CaOV3 (vector) cells.



Supplementary figure S1

Depletion of Bid inhibits TRAIL-induced apoptosis

We examined the effect of Bid depletion by RNA interference on TRAIL-induced apoptosis in TRAIL-sensitive OVCAR3 cells. Knockdown of Bid by siRNAs decreased the levels of Bid at 24 and 48 h, whereas, the control siRNA (siRNA Luc) did not affect Bid expression at 48 h (Figure 8a). Bid depletion completely inhibited TRAIL-induced cell death, as evident, by the same number of colonies found in long-term assays in the presence or absence of TRAIL (20 ng/ml) (Figure 8b). Apoptosis was also significantly reduced, ($P<0.001$) as shown by further limited oligosomal DNA fragmentation (Figure 8c) and lower percentage of hypodiploid cells, in the presence of Bid siRNA (Figure 8d). Consistent with these observations, we were unable to detect tBid by immunoblot in Bid siRNA-transfected cells whereas tBid was detected with the control siRNA (Figure 8e). We tried to perform similar experiments in CaOV3 cells, but were not able to obtain consistent downregulation of Bid in these cells. This was probably related to the fact that CaOV3 cells are relatively resistant to siRNA transfection, as we did not achieve efficient transfection with the control fluorescent siRNA (siRNA Luc), either. In all, our data suggest that Bid downregulation increased the resistance of OVCAR3 cells to TRAIL-induced apoptosis.

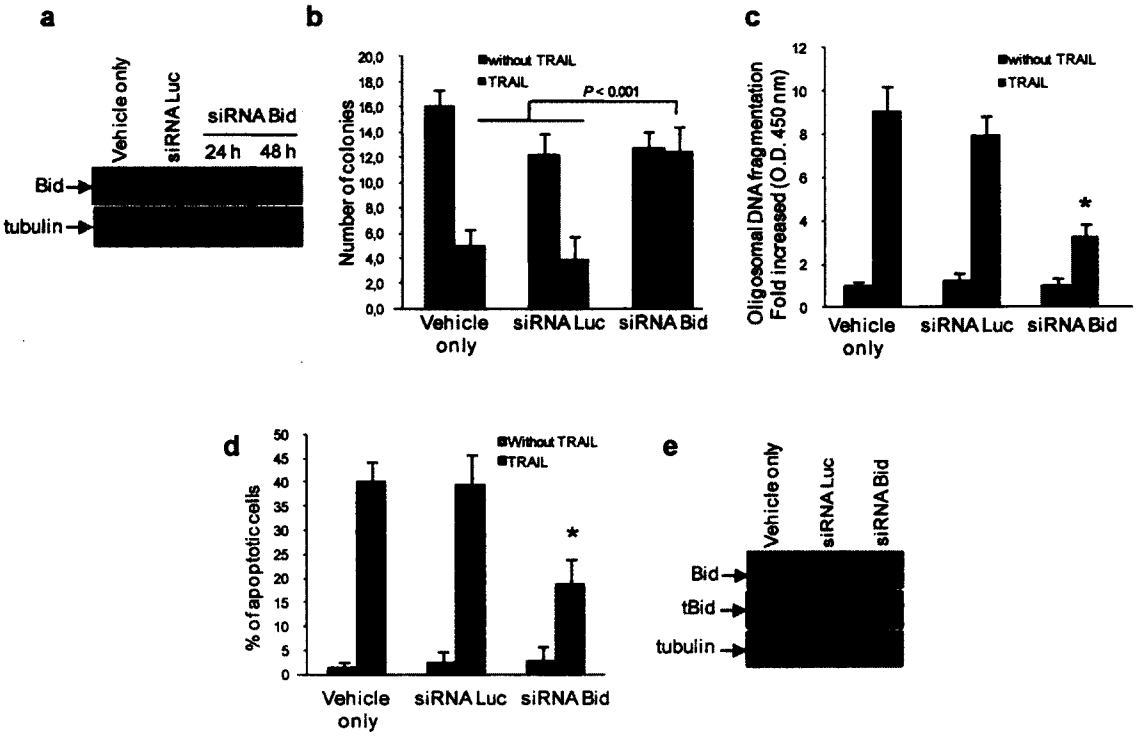


Figure 8 *Bid depletion inhibits TRAIL-induced apoptosis.* **(a)** Expression of Bid analyzed by western blot in OVCAR3 cells incubated with transfectant only (vehicle), control siRNA (siRNALuc) for 48 h or Bid siRNA and cell lysates were obtained at 24 and 48 h after transfection. Bid expression was assessed by western blot. **(b)** 48 h after transfection, TRAIL (20 ng/ml) was added for 24 h, after which the number of colonies was determined by staining with crystal violet after 14 days. OVCAR3 cells were left untreated or treated with TRAIL (20 ng/ml) 48 h after the transfection with siRNAs and apoptosis was measured by determining oligosomal DNA fragmentation 24 h after the addition of TRAIL **(c)** or by assessing the percentage of hypodiploid cells **(d)**. Data shown are representative of three independent experiments. Values are the means ± s.e.m. *P<0.001. **(e)** Bid cleavage in OVCAR3 cells transfected with siRNA was determined by western blot.

Bid overexpression promotes TRAIL-induced apoptosis in resistant cells

The requirement of Bid for TRAIL-induced apoptosis was confirmed by comparing the effects of stably expressing green fluorescent protein (GFP)-tagged Bid and GFP only in SKOV3ip1 cells. TRAIL treatment induced the cleavage of GFP-tagged Bid as shown by immunoblot (Figure 9a). However, the cleavage of endogenous Bid could not be detected as previously shown (Figure 6). TRAIL treatment also induced greater apoptosis in SKOV3ip1 cells overexpressing Bid compared with control cells (25.1±1.3 vs 12.7±0.8%, Figure 9b). Interestingly, the addition of LY294002 enhanced TRAIL-induced apoptosis in Bid-expressing SKOV3ip1 cells (35±1.5 vs 25.1±1.3%) suggesting that the inhibition of Akt phosphorylation enhanced Bid cleavage or that it alleviate a downstream blockade. The basal level of apoptosis in Bid-expressing SKOV3ip1 was unaffected by LY294002.

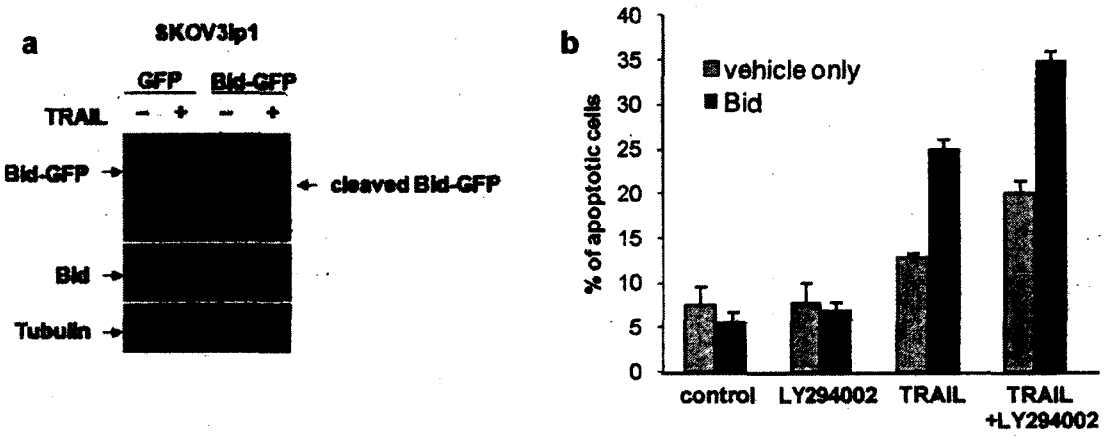


Figure 9 *Overexpression of Bid in resistant cells increases TRAIL-induced apoptosis.* TRAIL-resistant SKOV3ip1 cells were stably transfected with GFP expression vector (GFP) or with GFP-tagged Bid vector (Bid-GFP). **(a)** After selection, cells were analyzed for Bid expression and cleavage in the presence or absence of TRAIL (100 ng/ml) by immunoblot. **(b)** The percentage of apoptosis was measured as percentage of hypodiploid cells assessed by flow cytometry analysis during exposure to TRAIL (100 ng/ml) in the presence or absence of LY294002 (5 μM) for 24 h.

TRAIL resistance is not affected by XIAP downregulation

Bid and XIAP are substrates for caspase-3. In the presence of activated caspase-3, the full length 53 kDa XIAP protein is depleted and concomitant generation of a 30 kDa fragment occurs (Deveraux *et al.,* 1999). XIAP, the most potent caspase inhibitor of the IAP family, has been shown to regulate drug- and death receptor-induced apoptosis in EOC cells (Asselin *et al.,* 2001; Lane *et al.,* 2006). XIAP binds to the active form of caspase-3, but not to pro-caspase-3, and promotes its proteasomal degradation (Griffith *et al.,* 1998; Deveraux *et al.,* 1999; Lane *et al.,* 2006). As XIAP regulates caspase-3 activity, it may also affect Bid cleavage. We therefore compared the levels of XIAP expression in sensitive and resistant cells and in CaOV3-Akt-1 (wt) cells. There was no difference in XIAP expression between the cell lines (Figures 10a and b). As expected, XIAP was cleaved in TRAIL-sensitive CaOV3 cells but not in SKOV3ip1 cells (Figure 10c). To evaluate more specifically its contribution to TRAIL resistance, we downregulated XIAP expression with siRNA in SKOV3ip1 cells (Figure 10d). This treatment efficiently abolished XIAP expression but did not substantially sensitize cells to TRAIL (Figure 10e). In addition, XIAP downregulation had no effect on the cleavage of Bid and TRAIL-induced caspase-3 activation remained unaffected (Figure 10f). These data suggest that XIAP do not significantly contribute to TRAIL resistance in SKOV3ip1 cells and thus, support our concept that Akt interfere with Bid processing upstream of the mitochondria.

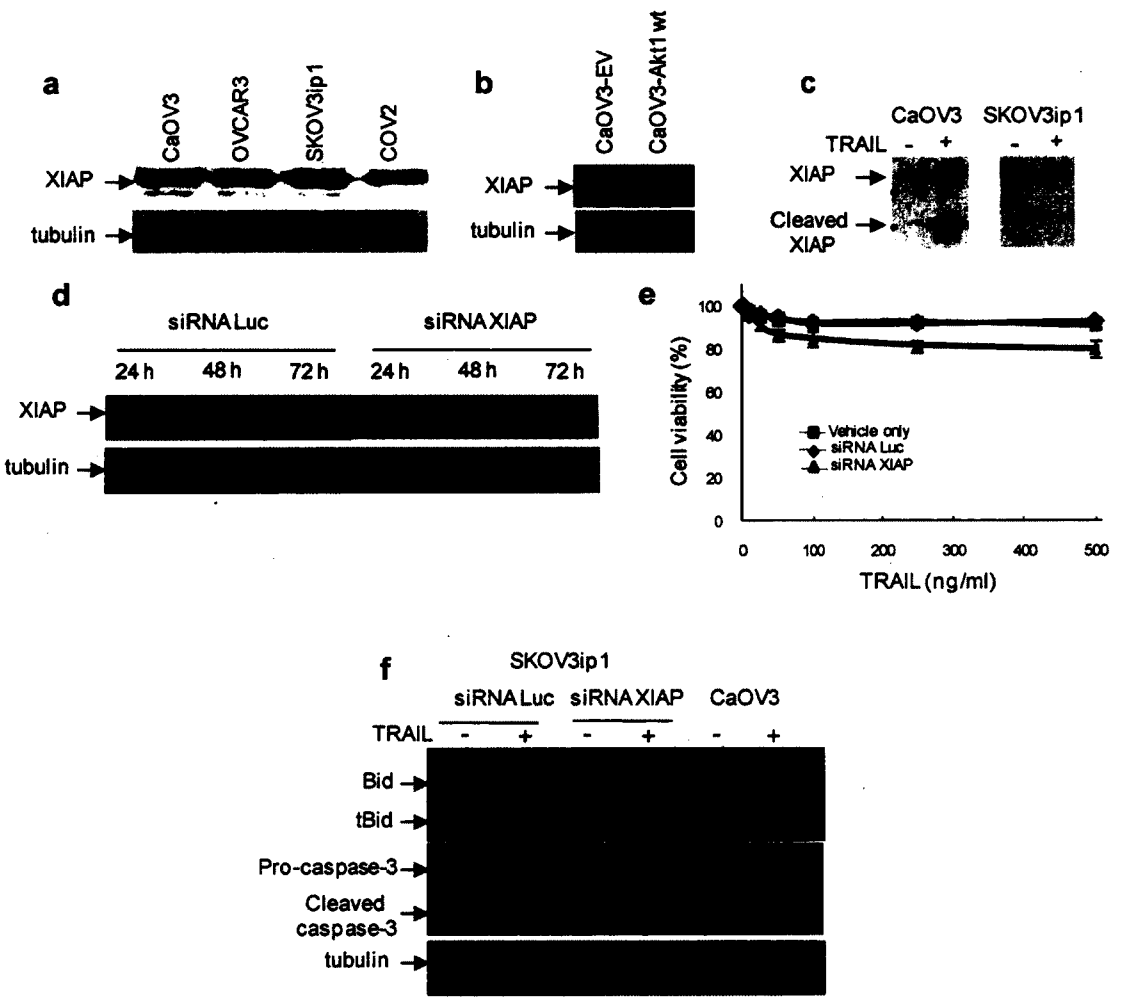


Figure 10 *Effect of X-linked inhibitor of apoptosis (XIAP) depletion on TRAIL-induced apoptosis.* **(a)** XIAP expression in sensitive and resistant cells determined by western blot. **(b)** XIAP expression in CaOV3-vector and CaOV3-Akt1 wt cells evaluated by Western blot. **(c)** CaOV3 and SKOV3ip1 cell were left untreated or were treated with TRAIL (100 ng/ml) for 24 h. Cell lysates were analyzed by western blot for XIAP expression. **(d)** SKOV3ip1 cells were transfected with control or XIAP siRNA. Cell lysates were obtained at 24, 48 and 72 h and XIAP expression was determined by western blot. **(e)** Transfected SKOV3ip1 cells were left untreated or were treated with increasing concentration of TRAIL for 48 h. Cell viability was assessed by XTT assay after 48 h. Data shown are representative of four independent experiments. Values are the means ± s.e.m. **(f)** Transfected SKOV3ip1 were left untreated or were treated with TRAIL (100 ng/ml) for 24 h. Cell lysates were analyzed for evidence of Bid cleavage and caspase-3 activation by western blot.

DISCUSSION

In this study, we show that the PI3K/Akt signaling pathway regulates TRAIL-induced apoptosis in EOC cells. Akt activation mediates TRAIL resistance by decreasing Bid expression and possibly by inhibiting its cleavage. We also showed that Akt activation correlates with decreased TRAIL sensitivity in primary cultures of ovarian carcinoma suggesting the importance of Akt activation in clinical setting.

Because of the potential of TRAIL in antitumor treatment, considerable effort is being directed at understanding the determinant of TRAIL resistance in cancer cells. In addition, many tumor cells, and in particular EOC cells, appear, at least *in vitro,* resistant to TRAIL-induced apoptosis (Lane *et al.,* 2004). Activation of the PI3K/Akt pathway is common in ovarian carcinoma (Bast *et al.,* 2009). There have been several reports showing the role of Akt in EOC drug resistance (Page *et al.,* 2000; Hu *et al.,* 2002; Fraser *et al.,* 2003; Westfall and Skinner, 2005; Yang *et al.,* 2006; Mabuchi *et al.,* 2007). However, the role of Akt in promoting survival against death receptor-induced apoptosis in EOC cells has not been clearly defined. A few reports suggested a role for the PI3K/Akt pathway in mediating TRAIL resistance. In two of these studies, Akt activation mediated by EOC ascites or serum from ovarian cancer patients inhibited TRAIL-induced apoptosis (Kang *et al.,* 2004; Lane *et al.,* 2007). Conversely, the inhibition of Akt phosphorylation in TRAIL-resistant SKOV3 cells enhanced TRAIL-induced apoptosis (Kim and Lee, 2005; Lane *et al.,* 2008). Taken together, these studies suggested a role for Akt in the regulation of TRAIL-induced apoptosis. This study shows that overexpression of Akt1 in TRAIL-sensitive EOC cells, CaOV3 enhanced resistance to TRAIL whereas inhibition of PI3K or Akt decreased resistance to TRAIL-mediated cell death. Therefore, our data clearly established that the PI3K/Akt pathway has an important role in regulating TRAIL-induced apoptosis in EOC cells. Intrinsic resistance to TRAIL among ovarian cancer cells appears to be mediated, at least in part, by activation of this pathway. The clinical relevance of this observation was confirmed in primary cultures of tumor obtained from women suffering with advanced ovarian carcinoma. We established a statistical correlation between the levels of Akt phosphorylation and TRAIL-IC_{50} in the primary cultures.

The anti-apoptotic effect of Akt is believed to occur downstream of caspase-8 activation. Similarly, we found that Akt activation or its inhibition did not interfere with the recruitment and processing of pro-caspase-8 at the DISC. Activation of Akt inhibits apoptosis before mitochondrial cytochrome c release (Kandel and Hay, 1999). To promote release of cytochrome c, tBid translocates to the mitochondria where, in concert with pore-forming Bax and Bak proteins, it activates the mitochondria (Luo *et al.*, 1998). Although it is well established that Akt may function at the mitochondria by phosphorylating Bad which prevents its interaction with anti-apoptotic proteins Bcl-2/Bcl-XL, there have been a number of reports, suggesting that Akt acts upstream of the mitochondria, although the site of action of Akt in the apoptotic cascade was not certain. In one study, Akt activation did not prevent Bid cleavage but inhibited Bak oligomerization and Bax activation, which are critical events in tBid-induced apoptosis (Majewski *et al.*, 2004). In another report, although the authors could not identify the precise mechanism by which tBid was unable to activate Bax or Bak in TRAIL-resistant colon cancer cells despite the fact the tBid effectively translocated to the mitochondria (Ndozangue-Touriguine *et al.*, 2008). In this study, we showed that Akt activation prevents the accumulation of caspase-8-induced tBid in TRAIL-resistant EOC cells, as shown by the observation that Akt overexpression in TRAIL-sensitive EOC cells prevents the detection of tBid. The fact that overexpression of Bid in SKOV3ip1 could induce apoptosis suggests that there was simply insufficient production of endogenous tBid after TRAIL treatment to directly activate Bak in these cells. The mechanism by which Akt interferes with the accumulation of tBid is not certain. Bid can be phosphorylated by casein kinase II leading to resistance to cleavage by caspase-8 and resistance to TRAIL-induced apoptosis (Desagher *et al.*, 2001; Izeradjene *et al.*, 2005). However, analyses of Bid phosphorylation by immunoblot in CaOV3 (vector) and CaOV3-Akt-1 (wt), showed that ectopic Akt1 expression did not affect Bid phosphorylation (N. Goncharenko-Khaider, unpublished data) suggesting that Akt pathway does not block Bid cleavage by interfering with its phosphorylation. In addition, TRAIL-induced apoptosis in resistant EOC cells was not affected by the chemical inhibition of casein kinase II with apigenin or with emodin (Lane D, unpublished data). It seems unlikely therefore that Akt affects Bid cleavage by interfering with Bid phosphorylation or by modulating the activity of casein kinase II. We cannot rule out the possibility that Akt

does not directly interfere with Bid cleavage, but instead regulates tBid degradation or acts downstream of Bid to antagonize tBid activity at the mitochondria.

The anti-apoptotic protein Mcl-1, a member of Bcl-2 family, has emerged as an important regulator of TRAIL sensitivity (Kim *et al.,* 2008). Mcl-1 can bind to pro-apoptotic BH3-only proteins, such as Bim, Puma, and Bak, thereby inhibiting the activation of the mitochondria (Cheng *et al.,* 2005). We have previously shown that ascites-induced Akt activation in CaOV3 cells does not affect the levels of pro-apoptotic proteins Bax and Bak and anti-apoptotic proteins Bcl-2 and Bcl-XL (Lane *et al.,* 2007). Consistent with these results, we showed in this study that expression of Mcl-1, Bcl-2 and Bcl-XL did not correlate with resistance to TRAIL.

Previous reports have suggested that in sensitive CaOV3 and HeLa cells, inhibition of TRAIL-induced apoptosis by Akt activation occurs by an upregulation of c-FLIP expression (Kang *et al.,* 2004; Lane *et al.,* 2007). Interestingly, we showed here that c-FLIP expression did not correlate with TRAIL sensitivity and c-FLIP expression was not affected by Akt inhibition in resistant cells. These data suggest that the mechanism by which Akt blocks the TRAIL signaling cascade may differ in sensitive vs resistant EOC cells. Akt activation in sensitive EOC cells may involve a dual resistance to TRAIL: a block at the DISC level through ascites-mediated upregulation of c-FLIPs (Lane *et al.,* 2007) and a block at the Bid level because we showed that Akt1 overexpression in sensitive CaOV3 cells decreased Bid protein levels. In some context, XIAP has been shown to be a critical regulator of death receptor-induced apoptosis (Jost *et al.,* 2009). Although the basal levels of XIAP were mostly comparable between sensitive and resistant cell lines, TRAIL treatment induced the cleavage of XIAP in sensitive cells, indicating that it is a consequence of cell death. However, the depletion of XIAP by siRNAs did not affect TRAIL-induced cell death, Bid levels and caspase-3 activation in SKOV3ip1 cells, ruling out a prominent role for XIAP in mediating TRAIL resistance.

In summary, we propose that binding of TRAIL to its cognate receptors induces caspase-8 activation at the DISC in both sensitive and resistant cells. In resistant cells, Akt

activation decreases Bid protein levels and possibly interferes with its cleavage. The level of tBid generated is therefore below a threshold that is insufficient to trigger Bid-mediated Bax or Bak activation. The lack of efficient mitochondrial activation prevents the mitochondrial feedback loop required to fully activate caspase-8 in the cytosol. However, the low level of Akt activation in sensitive cells is insufficient to decrease Bid levels or to prevent tBid accumulation. In these conditions the amount of tBid produced is sufficient to fully activate the formation of Bax/Bak complexes resulting in activation of the mitochondria.

MATERIALS AND METHODS

Ovarian cancer tumor samples and cell culture

Tumor cells were isolated from EOC ascites fluids as described previously (Lane *et al.*, 2004). Informed consent was obtained from all participants for this institutional review board approved protocol. All samples were supplied by the Tumor Bank of the Réseau de Recherche en Cancer du Fond de Recherche en Santé du Québec, Canada. All tumor cell samples were used at low passage (<10). The human EOC cell line CaOV3 and OVCAR3 were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 (Wisent, St-Bruno, QC, Canada) supplemented with 10% fetal bovine serum and insulin (10 mg/l). CaOV3 cells were cultured in Dulbecco's modified Eagle medium/F12 (Wisent) supplemented with 10% fetal bovine serum and 2mM glutamine. COV2 cells were derived from ascetic fluids obtained from a patient suffering with a primary stage III serous ovarian carcinoma and were maintained in Dulbecco's modified Eagle medium/F12 (Wisent) supplemented with 20% fetal bovine serum. The SKOV3ip1 human EOC cell line was kindly provided by J. Price (MD Anderson Cancer Center, Houston, TX, USA) and maintained in Dulbecco's modified Eagle medium/F12 supplemented with 10% fetal bovine serum (Wisent).

Reagents

TRAIL was purchased from PeproTech (Rocky Hill, NJ, USA). HRP-conjugated anti-mouse and -rabbit antibodies were purchased from Cell Signaling (Pickering, ON, Canada). Akt, caspase-3, caspase-9, COX IV, Bid and cytochrome c antibodies were also purchased from Cell Signaling. Antibodies for phospho-Akt (Ser-473) were obtained from Invitrogen (Biosource, Burlington, ON, Canada). Caspase-8 antibodies were obtained from R&D Systems (Minneapolis, MN, USA). Phenazine methosulfate and anti-tubulin antibody were obtained from Sigma. XTT reagents were from Invitrogen (Burlington, ON, Canada). LY294002 and Akt-specific inhibitor 1L-6-hydroxymethyl-chiro-inositol 2(R)-2-O-methyl-3-O-octadecylcarbonate were purchased from Sigma. Lipofectamine 2000 was purchased from Sigma (Oakville, ON, Canada).

Retroviral preparation and transduction efficiency

The pLPCXL retroviral vector construct encoding wild type (wt) Akt1 was kindly provided by N. Rivard (University of Sherbrooke, Québec, Canada). Retroviral particles were generated by transfecting 293T cells with pLPCXL-Akt1, pVPack-VSV-G and pVPack-GP (Invitrogen) and used to infect CaOV3 cells. Pools of productively infected cells obtained by selection with puromycin (0,4 mg/ml) for 10 days were used for further analysis. In the case of stable expression of Bid, SKOV3ip1 cells were transfected with either pEGFPN1 vector (Clontech, Mountain view, CA, USA) or pEGFPN1 containing full-length Bid (cloned in EcoR1 site), selected in G-418 (1 mg/ml) for 10 days, and then used as a pooled.

Quantitative RT–PCR

Total RNA was extracted from CaOV3 cells using TRIzol reagent according to the manufacturer's protocol and subjected to reverse transcription using Promega (Madison, WI, USA) reverse transcriptase enzyme. The integrity of the cDNA was assessed by SRBR-base quantitative PCR, done on four housekeeping genes: MRPL19, PUM1, GAPDH and actin. Each sample was normalized to housekeeping gene levels. Primer

sequences are available on request. Cycle conditions for all PCRs were as follows: an initial incubation of 2 min at 95 °C followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s. The amplification was carried out by a 2 min incubation at 72 °C. PCR products quantification was performed as previously described (Klinck *et al.*, 2008).

Cell viability assays

Short-term cell viability in the presence or absence of TRAIL was determined by XTT assay. Briefly, cells were plated at 20 000 cells/well in 96-well plates in complete medium. The next day, cells (confluence 60–70%) were treated with human TRAIL and incubated for 48 h. At the termination of the experiment, the culture media was removed and a mixture of phosphate-buffered saline and fresh media (without phenol red) containing phenazine methosulfate and XTT was added and kept for 30 min at room temperature. The absorbance was determined using a microplate reader at 450nm (TecanSunrise, NC, USA). The percentage of cell survival was defined as the relative absorbance of untreated versus TRAIL-treated cells. For clonogenic survival assays, cells were plated in six-well tissue culture plates in standard medium. The next day, cells were treated with TRAIL (20–100 ng/ml) for 24 h, followed by several washes with standard medium. Then, cells were incubated in standard medium for 14 days. Cells were fixed and stained with crystal violet. The number of colonies, consisting of >25 cells, in triplicate was counted.

Apoptosis and caspase assays

Caspase-3 and caspase-8 fluorogenic protease assays were performed according the manufacturer's protocol (R&D Systems). In brief, 3×10^6 cells were lysed in 250 µl of cell lysis buffer, and total cell lysates were incubated with 50 µM of DEVD-AFC (caspase-3) or IETD-AFC (caspase-8) substrate for 1 h at 37 °C. Caspase-3 and caspase-8 activities were measured on a Versa Fluor fluoremeter (BioRad, Hercules, CA, USA). Protein concentration of the lysates was measured with Bio-Rad protein assay kit according to the manufacturer's recommendations. The release of nucleosomal DNA into the cytoplasm as a measure of apoptosis was determined using the Cell Death Detection ELISA Kit (Roche,

Laval, Québec, Canada) according to the manufacturer's instruction. The absorbance was determined using a microplate reader at 410 nm (TecanSunrise). Propidium iodide staining for DNA fragmentation was done by fixing cells and staining them with propidium iodide for DNA analysis content as previously described (Lane *et al.,* 2006). A total of 10 000 events were analyzed by flow cytometry and the percentage of hypodiploid cells was measured.

Western blot analysis

Cells were harvested and washed with ice-cold phosphate buffered saline. Whole cell extracts were prepared in lysing buffer (glycerol 10%, Triton X-100 1%, TRIS 10mM pH 7.4, NaCl 100mM, EGTA 1mM, EDTA 1mM, $Na_4P_2O_7$ 20mM, NaF 1mM, Na_3VO_4 2mM and SDS 0.1%) containing protease inhibitors (0.1mM AEBSF, 5 μg/ml pepstatin, 0.5 μg/ml leupeptin and 2 μg/ml aprotinin) and cytosolic proteins were separated by 12% SDS–PAGE gels. Lysates for phosphorylated proteins were prepared in the presence of phosphatase inhibitors (100mM sodium fluoride, 100 μM sodium pyrophosphate and 250 μM sodium orthovanadate). Proteins were transferred to PVDF membranes (Roche) by electroblotting, and immunoblot analysis was performed as previously described (Lane *et al.,* 2006). All primary antibodies were incubated overnight at 4 °C. Proteins were visualized by enhanced chemiluminescence (GE Healthcare, Baie d'Urfé, Québec, Canada).

Knockdown of Bid and XIAP

The Fluorescein-labeled Luciferase GL2 duplex was used as a control, the Bid siRNA was obtained from Dharmacon Research (Lafayette, CO, USA) and XIAP siRNA (Signal Silence XIAP siRNA) were purchased from Cell Signaling. Cells (6×10^4) were seeded in six-well plates and allowed to adhere for 24 h. Cells (50% confluent) were transfected with a mixture containing Lipofectamine 2000 (Invitrogen Life Technologies, Burlington, ON, Canada), optiMEM (Invitrogen) and Bid siRNA (10 μM) or XIAP siRNA (25 μM). The siRNAs/oligofectamine mixture was then added to the media of six-well plates containing

cells. Cells were incubated for 4–6 h at 37 °C in a CO_2 incubator and a medium containing fetal bovine serum was then added to it. After 48 h, TRAIL was added and cells were further incubated for another 48 h after which XTT assays were performed. For western blotting, the cells were lysed at 24 or 48 h post-transfection and total cell lysates were analyzed by immunoblot.

Statistical analysis

Statistical comparisons between two groups were performed using the Student's t-test and with ANOVA when comparing the data with more than two treatments groups. Statistical significance was indicated by $P<0.05$. The strength of the association between TRAIL IC_{50} and Akt phosphorylation levels in primary tumor samples was measured using Pearson's correlation coefficient parametric approach.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Discussion and conclusions

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent as it selectively kills tumor cells but spares normal cells. Resistance to TRAIL among ovarian cancer tumor cells could limit its therapeutic use. In the present study, we have investigated the mechanisms of intrinsic resistance to TRAIL among EOC cells.

We have previously tested the effect of this agent on the panel of different EOC cell lines and primary samples and demonstrated that they have variable sensitivity to TRAIL (LANE *et al.,* 2004). The cell lines were subdivided into TRAIL-sensitive (<20% cell viability) and TRAIL-resistant (80% cell viability) based on an XTT cell viability assay. For this study, we selected two TRAIL-sensitive cell lines (CaOV3 and OVCAR3) and two TRAIL-resistant cells (SKOV3ip1 and COV2). We found that TRAIL-resistant cell lines have the highest basal activity of the Akt protein kinase. Because Akt is known to block apoptosis, we tested whether the inhibition of this pathway in TRAIL-resistant cell lines and Akt overexpression in TRAIL-sensitive cell lines would affect the sensitivity of these cells to TRAIL. We found that the treatment of TRAIL-resistant cells with PI3K inhibitor LY294002 or a specific Akt inhibitor sensitized these cells to TRAIL-induced apoptosis. Overexpression of Akt1 in TRAIL-sensitive CaOV3 cells rendered them more resistant to TRAIL. These data suggested that Akt is a critical mediator of resistance to TRAIL in EOC cells. To further confirm the clinical relevance of these observations, we established a statistical correlation between the levels of Akt activation and the TRAIL-IC_{50} in the primary cultures of tumor cells obtained from women with advanced ovarian carcinoma and demonstrated that TRAIL-resistance correlates with higher levels of Akt activation.

To identify which step of TRAIL-mediated apoptotic pathway is blocked in TRAIL-resistant EOC cell lines, we investigated different steps of the apoptotic pathway activation in our TRAIL-resistant and –sensitive cell line models. Altered expression of the death receptors TRAIL-DR4 and –DR5 has been previously associated with resistance to TRAIL in some tumor cells. Several studies have demonstrated high expression of death receptors in TRAIL-sensitive tumor cells (FRANK *et al.,* 1999; ZHANG and ZHANG, 2008). In

contrast, low or undetectable levels of death receptors DR4 have been found in resistant cancer cells (KIM *et al.*, 2000). However, we found that expression of TRAIL death and decoy receptors at the cell surface of sensitive and resistant EOC cell lines was similar. In addition, endogenous expression of FADD and a protease-deficient homolog c-FLIPs and c-$FLIP_L$ did not correlate with TRAIL sensitivy (LANE *et al.*, 2004). Thus, in ovarian cancer cells, intrinsic TRAIL resistance does not appear to be mediated by alterations in death receptor expression.

Further, we investigated the pro-caspase-8 activation at the DISC. Pro-caspase-8 typically exists as a monomer in cytosol, but upon DISC formation, it undergoes dimerization. The dimerization of two monomers of pro-caspase-8 (p55/p55) results in a conformational change, which exposes the active site of the caspase (MUZIO *et al.*, 1998). Pro-caspase-8 cleaves itself between p18 and p10 domains forming heterotetrameric complex (p43-p10/p43-p10). This complex undergoes the second cleavage between p26 and p18 which frees the final product of caspase-8 (p18-p10/p-18-p10) (CHANG *et al.*, 2003). Cleaved caspase-8 then recognizes its downstream substrates (caspase-3 and pro-apoptotic protein Bid). We were able to detect the full length pro-caspase-8 (p55) and the processed caspase-8 fragment (p43) at the DISC by immunoprecipitation in both TRAIL-sensitive and TRAIL-resistant cell lines suggesting that pro-caspase-8 is recruited and processed at the DICS in both cell line models. However, we were not able to detect the mature activated caspase-8 fragment (p18/p10) at the DISC. When we assessed caspase-8 activation by fluorometric assay, we detected caspase-8 activation in both TRAIL-sensitive and –resistant cells, even though the amount of activated caspase-8 was lower in TRAIL-resistant cells. Several studies have reported difficulty in detecting active caspase-8 at the DISC in type II cells (SCAFFIDI *et al.*, 1998; SCAFFIDI *et al.*, 1999; SCHUG *et al.*, 2010). Interestingly, it was recently demonstrated that activated caspase-8 is associated with mitochondrial membrane which is dependent on presence of the mature cardiolipin, where it forms macromolecular complex with Bid within which the cleavage of Bid occurs. This provides a means by which concentration of enzyme (caspase-8) and its substrate (Bid) occurs on the organelle where it is needed (mitochondria) (SCHUG *et al.*, 2010). If indeed, upon its cleavage caspase-8 translocates and localizes at the mitochondria, it could

explain why we did not detect activated caspase-8 at the DISC. Based on these results, we concluded that the DISC formation and the caspase-8 activation are not blocked in TRAIL-resistant EOC cell lines.

EOC cells are classified as type II cells, meaning that in these cells DISC assembly is slower and production of caspase-8 is lower. In this scenario, caspase-8 must engage the intrinsic (mitochondrial) pathway to amplify the death signal and execute apoptosis (OZOREN and EL-DEIRY, 2002). The transition from extrinsic pathway to the intrinsic pathway is achieved through caspase-8-mediated processing of Bid and the produced truncated Bid (tBid) shifts to a separate complex, containing Bax/Bak. Activated Bax and Bak undergo conformational changes, resulting in formation of giant pores in the outer mitochondrial membrane. As a result, cytochrome c is released in cytosol (WEI *et al.*, 2000). We have found that TRAIL-induced cytochrome c release was blocked in TRAIL-resistant cells, but not in TRAIL-sensitive. Furthermore, we did not detect caspase-9 or caspase-3 activation in TRAIL-resistant cells whereas in TRAIL-sensitive cells these caspases were activated upon treatment with TRAIL. Thus, Akt blocks apoptosis in TRAIL-resistant EOC cell lines downstream of the DISC but upstream of the mitochondria (Figure 32). There have been several reports to suggest that Akt acts upstream of the mitochondria to regulate cell death by TRAIL, although the site of action of Akt in the cell death pathway was not well established (KANDASAMY and STRIVASTAVA, 2002; NESTEROV *et al.*, 2001; GIBSON *et al.*, 2002). The mitochondrial cell death pathway is regulated by the interaction between pro- and anti-apoptotic members of the Bcl-2 family. Consequently, we investigated the endogenous expression of pro-apoptotic protein Bid and demonstrated that Bid expression is significantly lower in TRAIL-resistant cell lines compare to sensitive. Importantly, we have shown that levels of the pro-apoptotic Bid protein were negatively regulated by Akt, since overexpression of Akt1 in TRAIL-sensitive CaOV3 cells resulted in downregulation of Bid and inhibition of PI3K with LY294002 in TRAIL-resistant cells resulted in increased expression of Bid protein. In addition, we confirmed that Akt regulates Bid expression on transcriptional level, since Bid mRNA levels were decreased in CaOV3 cells overexpressing Akt1 and TRAIL-resistant cells show lower levels of Bid mRNA compare to TRAIL-sensitive cells. We also showed that

overexpression of Bid-GFP in TRAIL-resistant cells not only induced cell death, but also resulted in cleavage of Bid-GFP. Interestingly, inhibition of PI3K with LY294002 in SKOV3ip1 cells with forced Bid expression resulted in even more increased TRAIL-induced apoptosis. In addition, knockdown of Bid by siRNA in TRAIL-sensitive cells inhibited TRAIL-induced Bid cleavage and decreased sensitivity of these cells to TRAIL. Taken together, these results demonstrated that Bid is a critical downstream target for Akt-mediated TRAIL resistance.

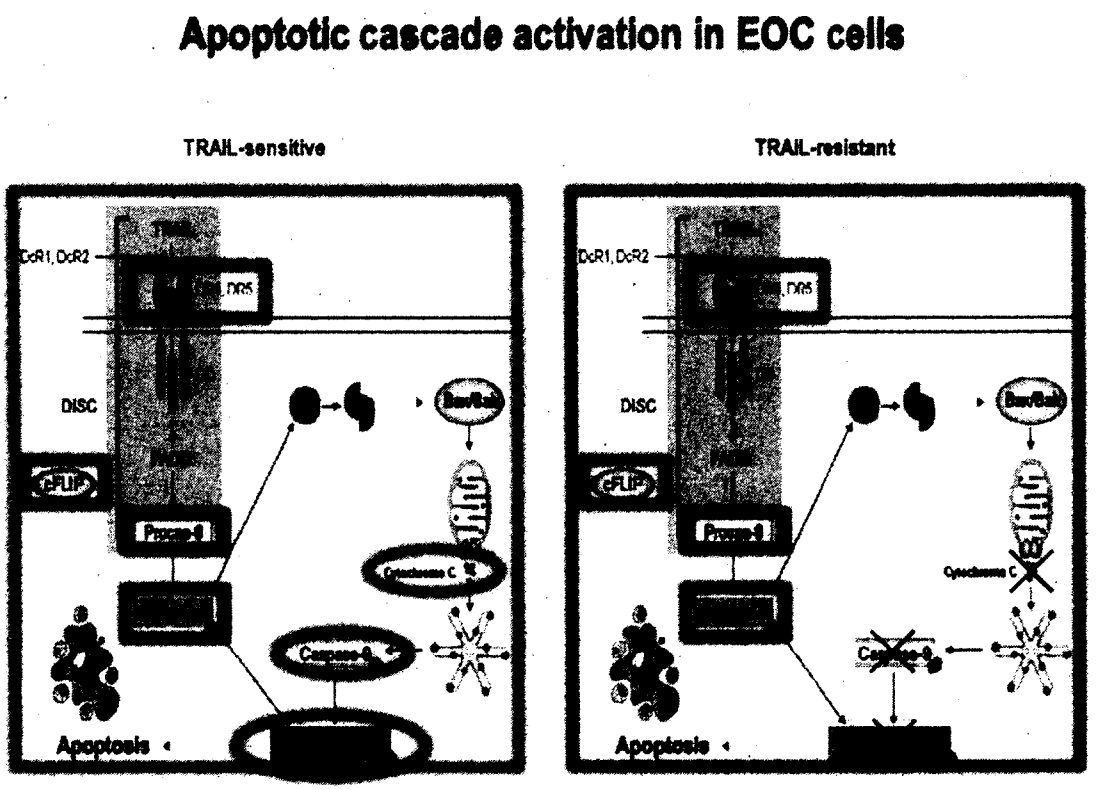

Figure 32. Apoptotic cascade activation in EOC cells. Schematic representation of the apoptotic cascade in sensitive and resistant cells. Apoptotic molecules that are inserted into a blue rectangle were not associated with TRAIL resistance. Molecules inserted into a green oval have been found to be activated upon TRAIL treatment. Red X represents blockade in apoptotic pathway steps.

Since Bid is converted into its active form by caspase-8-mediated cleavage, we investigated Bid cleavage in our cell line models. We did not detect Bid cleavage in TRAIL-resistant EOC cells, whereas in TRAIL-sensitive cells we observed depletion of the full length Bid and appearance of tBid after 2h of exposure to TRAIL. In addition, we showed that overexpression of Akt1 in TRAIL-sensitive CaOV3 cells resulted in the blockade of Bid cleavage and the inhibition of the PI3K with LY294002 in TRAIL-resistant cells resulted in the depletion of full length Bid, although we did not detect tBid on Western blot. One possible explanation for the lack of tBid detection is the fact that there could be a very low level of tBid produced in TRAIL-resistant cells that is not detectable by Western blot, considering the fact that the endogenous level of Bid is very low in these cells. It has been reported that tBid appears to be a target for ubiquitin/proteasome system (BREITSCHOPF *et al.*, 2000). Another study demonstrated that upon Bid cleavage, the N-terminal fragment (tBid-N) is ubiquitinated and degraded, thus freeing the BH3 domain in the C-terminal fragment (tBid-C) and this unconventional ubiquitination and proteasome-dependent degradation of tBid-N is required to unleash pro-apoptotic activity of tBid-C (TAIT *et al.*, 2007). In addition, tBid was identified as a substrate of the ubiquitin ligase Itch, which can specifically interact with and ubiquitinate tBid, but not intact Bid. Itch can be positively regulated by the EGF pathway (AZAKIR *et al.*, 2010). EGFR tyrosine kinase receptor exerts many of its biological activities through the activation of the PI3K/Akt pathway (GAN *et al.*, 2010). This leads to the suggestion that removal of tBid by proteasomal degradation could result in disappearance of both Bid and tBid on our Western blot, although this remains to be confirmed. Thus, it would be interesting to investigate if the treatment of resistant cells with TRAIL in presence of proteasomal inhibitor would result in Bid cleavage and whether it would sensitize these cells to TRAIL. If the combined treatment with TRAIL and the proteasomal inhibitor in resistant cells increases the levels of tBid, then we could investigate if Akt is implicated in this effect by treating CaOV3 cells overexpressing Akt1 with proteasomal inhibitor and TRAIL and see if Bid cleavage is restored in these conditions and if these cells become more sensitive to TRAIL.

One would assume that since Akt is a protein kinase that can regulate activity of number of substrates by phosphorylation, it could directly phosphorylate Bid and possibly prevent its

cleavage. We assessed Bid phosphorylation in TRAIL-sensitive and –resistant cell lines, as well as in CaOV3 cells overexpressing Akt1 and did not find any differences in phosphorylated Bid expression (data not shown). In one of the study which aimed to identify the kinase(s) responsible for Bid phosphorylation, it was demonstrated that casein kinase I and II can phosphorylate Bid, but not other protein kinase such as Akt, PKA, PKC and MAPK. When phosphorylated by casein kinase I and II, Bid was insensitive to caspase-8 cleavage *in vitro* (DESAGHER *et al.*, 2001). However, we observed that TRAIL-induced apoptosis in resistant EOC cells was not affected by chemical inhibition of casein kinase II with apigenin or with emodin (data not shown). To investigate whether Akt can directly interact with Bid we performed immunoprecipitation of FLAG-tagged Akt with Bid. This experiment failed to demonstrate such an interaction. Although it remains unclear how the PI3K/Akt pathway inhibits Bid cleavage, our data suggests that it is not through direct interaction or through Bid phosphorylation.

It has been documented that Akt may regulate a variety of apoptotic molecules in multiple ways (PARCELLIER *et al.*, 2008). Therefore, it is possible that Bid is not the sole downstream target of Akt contributing to TRAIL resistance. It is well established that Akt may phosphorylate the pro-apoptotic protein Bad which prevents its interaction with anti-apoptotic Bcl-2 and Bcl-XL (DATTA *et al.*, 1997). We found that in CaOV3 cells overexpressing Akt1 the level of phosphorylated Bad is indeed higher compare to control empty vector expressing cells (data not shown). Although it remains to be proven, Bad phosphorylation may contribute to TRAIL resistance. In addition, we investigated whether Akt can regulate expression of other Bcl-2 family members apart from Bid. We assessed expression of different pro- and anti-apoptotic proteins in our TRAIL-resistant and –sensitive cell lines. We showed that expression of Bcl-2, Bcl-XL, Mcl-1, Bak, Bax, Bik, and Bad did not correlate with TRAIL-resistance.

Previous reports have demonstrated that XIAP, the potent caspase inhibitor, is a critical regulator of TRAIL-induced apoptosis (VOLGER *et al.*, 2007; JOST *et al.*, 2009). XIAP can directly bind to the caspase-3 and send it to proteasomal degradation. Activated caspase-3, in its turn, can cleave Bid and XIAP (HÖRNLE *et al.*, 2010; SLEE *et al.*, 2000).

Since XIAP negatively regulates caspase-3 and caspase-3 can increase Bid cleavage, we investigated if XIAP could also be important for TRAIL resistance in EOC. However, we found no difference in XIAP expression between sensitive and resistant cell lines. As expected, we observed XIAP cleavage in TRAIL-sensitive cells (because caspase-3 is activated in these cells upon treatment with TRAIL), but not in TRAIL-resistant. In addition, downregulation of XIAP in TRAIL-resistant cell line SKOV3ip1 had no effect on caspase-3 activation or Bid cleavage and it did not sensitize these cells to TRAIL-induced apoptosis (GONCHARENKO-KHAIDER *et al.*, 2010). These data demonstrated that XIAP does not contribute significantly to TRAIL resistance.

Conclusions

In this study, we provide evidence that regulation of the pro-apoptotic protein Bid by PI3K/Akt pathway is important for TRAIL-resistance in EOC cells. We propose that TRAIL-sensitive EOC cell lines respond to TRAIL by assembly of the DISC and activation of the caspase-8. Caspase-8 cleaves the pro-apoptotic protein Bid and production of tBid allows MOMP and cytochrome c release, activation of caspase-9 and downstream effector caspases -3, -6, -7 leading to cell death (Figure 32). TRAIL-resistant EOC cells respond to TRAIL in the following manner: DISC is assembled and caspase-8 is activated but in the smaller amount compared to TRAIL-sensitive cells. Akt activation in TRAIL-resistant cells results in downregulation of Bid and this negative regulation occurs on transcriptional level. Akt also interferes with Bid cleavage by unknown indirect mechanisms. As a result, the level of produced tBid is very low in these TRAIL-resistant cells which is insufficient to trigger cytochrome c release and activate apoptotic cascade downstream of mitochondria (Figure 33).

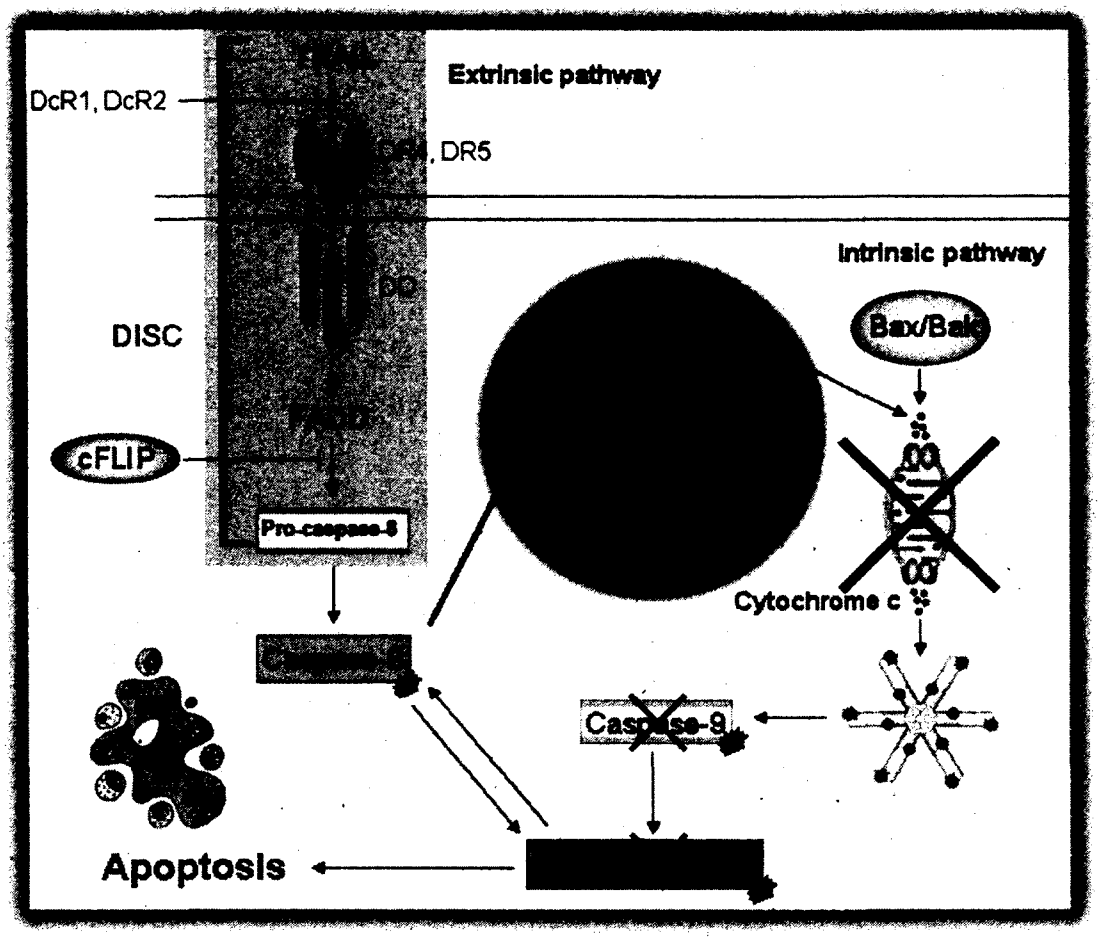

Figure 33. Proposed model of apoptotic cascade activation in TRAIL-resistant EOC cells. We propose that in TRAIL-resistant EOC cells binding of TRAIL to its receptors results in DISC assembly and caspase-8 activation but in the smaller amount compared to TRAIL-sensitive cells. Akt activation in TRAIL-resistant cells results in downregulation of pro-apoptotic protein Bid and this negative regulation occurs on transcriptional level. Akt also interferes with Bid cleavage by unknown indirect mechanisms. As a result, the level of produced tBid is very low in these TRAIL-resistant cells which is insufficient to trigger cytochrome c release and activate apoptotic cascade downstream of mitochondria.

Perspectives

We speculate that Akt activation may be a potential biomarker to predict patient's response to TRAIL therapy and that the inhibition of the PI3K/Akt pathway can become one of the strategies to overcome resistance to TRAIL therapy in ovarian cancer. We also propose that Bid protein could be targeted to reverse TRAIL resistance in epithelial ovarian cancer. Recently, Bid gene therapy has been tested against advanced lung cancer and has been shown to be safe and more effective with addition of cisplatin and dexamethasone (FUKAZAWA *et al.*, 2009).

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